# RESISTANCE TO AFLATOXIN ACCUMULATION IN MAIZE

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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August 2011

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Cornell University 2011

Aflatoxins are carcinogenic compounds produced by the fungal pathogen *Aspergillus flavus* and other *Aspergillus* spp. *A. flavus* infects maize (*Zea mays* L.) and other agricultural commodities. Regulation in developed countries permits only extremely low levels of aflatoxin in food, and as a result farmers are exposed to significant economic losses. In developing countries, where *A. flavus* populations are more prevalent and regulations are rarely enforced, aflatoxins cause significant health burdens for human populations. Management with the use of maize lines that are resistant to aflatoxin accumulation could benefit farmers around the world. Little is known about the factors contributing to resistance and its interaction with the environment. The objective of this dissertation was to better understand resistance to aflatoxin accumulation in maize so that this resistance can be incorporated into maize hybrids. A new technique for the evaluation of *A. flavus* colonization using quantitative real-time PCR (qPCR) was developed and validated. There was a strong correlation between colonization of *A. flavus*, as measured by qPCR, and aflatoxin levels. In addition to resistance to aflatoxin accumulation, variation was detected in maize for susceptibility to silk and kernel colonization. Resistance to aflatoxin accumulation was correlated with flowering time, and with kernel physical traits, such as fiber, ash, carbohydrate and seed weight. An analysis of the inheritance of resistance was conducted in the CML322 x B73 population. Moderate levels of heritability (63%) suggested that significant gains could be obtained from breeding with this population. Thirteen quantitative trait loci (QTL) for resistance to aflatoxin accumulation and other silk and kernel traits were found in three years of experiments.

One QTL with moderate effect in maize bin 4.08 was confirmed using near isogenic lines. A meta-analysis of QTL was conducted with all the reported QTL found in the literature including QTL for resistance to other ear rots. This meta-analysis indicated that QTL for multiple ear rot diseases co-localize. The analysis resulted in reduced confidence intervals, presumably increasing the feasibility of breeding strategies that utilize molecular markers.

# BIOGRAPHICAL SKETCH

Santiago was born and raised in Quito, Ecuador. During high school he developed a profound interest in applied biology and decided to learn how to produce more and better quality food. Thus, he earned a bachelor's degree in farm engineering at the Army Polytechnic School in Ecuador. After completing his bachelor's, he worked for a fresh flower production company as a farm manager. Later, following his interests in research and food production, he became a research assistant at the International Potato Center (CIP). At CIP, under the direction of Dr. Greg Forbes, Santiago was astonished by the damage a plant disease (potato late blight) can inflict on a crop. Thanks to Greg's contacts and recommendations, Santiago was able to move to the United States to start his graduate education in plant pathology. Santiago earned a master's degree in the soybean pathology lab with Dr. Anne Dorrance at The Ohio State University. In the fall of 2006, he moved to Ithaca to become a Ph.D. student with Dr. Rebecca Nelson. At Cornell, Santiago was able to match his interests in agricultural development and science by studying maize resistance to aflatoxin, a plant disease that inflicts a health burden on people.

Dedicado a mis padres Jorge y María Teresa.

ACKNOWLEDGMENTS

My Ph.D. dissertation would have been impossible without the advice, help and support of many people. First, I am extremely grateful to my advisor, Dr. Rebecca J. Nelson for making sure I was accepted into Cornell and then to her lab. I hope I have been able to capture at least some part of her ideas and advice in the execution of this dissertation.

A significant percentage of the work in this dissertation was conducted in the state of Mississippi with the invaluable help and support of all the personnel of the corn host plant resistance research unit from the USDA/ARS. Special thanks are due to the co-authors of the manuscripts that will come out of this dissertation: Dr. W. Paul Williams, Dr. Gary L. Windham and Dr. Marilyn Warburton. I am indebted to Marilyn for allowing me to stay in her house for three summers in Starkville. It is hard to imagine how I would have conducted field experiments in Mississippi without Marilyn's support.

I would also like to thank the Cornell community, especially Dr. Margaret Smith and Dr. Gary Bergstrom, members of my special committee for their advice during the realization of this dissertation. Thanks are also due to past and present members of the lab. Special thanks to Chia-Lin Chung, Jesse Poland, Lin-Si Hsieh, Jaci Benson, Samuel Mutiga, and Tiffany Jamann for invaluable discussions and help with *in-vitro* inoculation experiments. Many thanks also to undergraduates who helped in the realization of many experiments, including Wenzhe Li, Chris Mancuso, Nelson Chepkwony, Ariel Fialko, Oliver Ott, Sara Heinz, Kristen Kennedy, Kerri Lyons, Joy Longfellow, and Ellie Walsh. I also thank fellow graduate students in the

department of Plant Pathology and Plant-Microbe Biology, especially Paola Zuluaga and Jose Huguet for multiple discussions about my project. I am very thankful to Tiffany, my wife, for her support, encouragement and for complementing my life.

Finally, I would like to acknowledge the department of Plant Pathology and Plant-Microbe Biology, Cornell University and The McKnight Foundation for financial support.

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## *Introduction*

*Aspergillus flavus* Link:Fr is a soil-inhabiting fungus that is a weak opportunistic pathogen of plants and animals. It is the most common causal agent of Aspergillus ear rot of maize, but causes relatively little direct yield loss as a pathogen (73). *A. flavus* is of great concern because it produces toxic secondary metabolites, the most dangerous of which are aflatoxins. Several species of the genus *Aspergillus* produce aflatoxins, which are potent carcinogenic compounds affecting humans and animals at low doses. Aflatoxin accumulation varies with host genotype and environment. Despite the strong genotype-by-environment interaction, there are numerous reports of significant variation in the levels of aflatoxin accumulation among distinct maize lines (6, 25, 78, 84). This chapter reviews the literature with an emphasis on maize resistance as a means to manage *A. flavus* colonization and aflatoxin accumulation.

*A. flavus* is an ascomycete in the class Eurotiomycetes, subclass Eurotiomycetidae. This subclass is characterized by producing prototunicate asci in cleistothecia (23). Phylogenetic analysis of five gene regions has placed *A. flavus* in the clade Eurotiales together with *Penicillium* spp. Many Eurotiales are considered as aggressive saprobes, and characteristically are xerotolerant (tolerate extremely low

water activities), osmotolerant and thermotolerant. This group of fungi also contains the model organism *A. nidulans* (*Emericella nidulans*) (23). Molecular analysis shows that there are clearly two groups of *A. flavus* isolates. Within these groups there is a long history of reproductive isolation. The teleomorph (sexual stage) of *A. flavus* was recently described as *Petromyces flavus* (33). Interestingly, *A. oryzae*, used for soy sauce production, is monophyletic with *A. flavus* (22). In addition to aflatoxins, *A. flavus* and other *Aspergillus spp.* produce a wide array of mycotoxins such as cyclopiazonic acid, aflatrem and many other polyketides (48).

### *Aflatoxins*

*A. flavus, A. parasiticus, A. nominus, A. pseudotamarii, A. bombycis, A. ochraceoroseus* and *Emericella venezualensis* produce aflatoxins (88). Aflatoxins are polyketide-derived furanocoumarins that were first discovered in *A. flavus* after an outbreak of Turkey X disease in England (88). There are at least 15 aflatoxin intermediates in the pathway. Sterigmatocystin and dihydrosterigmatocysitin are close to the end of the pathway and are produced by the model organism *A. nidulans*. Four major aflatoxins are found in agricultural commodities: $B_1$, $B_2$, $G_1$ and $G_2$. Hydroxylated sub-products that are usually found in animals that have consumed contaminated food are known as aflatoxins $M_1$ and $M_2$.

Aflatoxin $B_1$ is the most potent naturally-occurring chemical liver carcinogen known. Mutagenesis occurs because a reactive oxygen derivative from the metabolism of aflatoxins in the liver binds to DNA, causing transversions and transitions (75). The ingestion of high doses of aflatoxin, usually from contaminated food such as maize, causes liver damage that can be fatal (26). Chronic exposure to aflatoxins has been implicated in immunosupression (76) and shown to produce

growth impairment in children (24). Exposure to low and high doses of aflatoxins can produce cancer (30, 77).

There is no known function of aflatoxins in *Aspergillus* spp. For *A. flavus*, production of aflatoxin on maize coincides with a switch in substrate availability from saccharides to triglycerides (44). The attenuation of aflatoxin with antioxidant compounds such as gallic acid led to the hypothesis that aflatoxigenenesis is a fungal response to oxidative stress (35, 38, 60). Using *in-vitro* assays, it was found that caffeic acid (an antioxidant) reduced aflatoxin by more than 95% compared to the control while fungal biomass remained the same. In addition, all the genes in the aflatoxin biosynthetic cluster were down-regulated in the caffeic acid treatment (38). However, a review of the effect of antioxidants shows that, although many inhibitors of aflatoxin production are antioxidants and inducers are oxidants, antioxidant capacity does not predict an effect on aflatoxins biosynthesis (31).

***The Problem for Agricultural Production***

Developed nations strictly regulate the amounts of aflatoxins present on food to low levels. For example, Canada and the United States allow maximum levels of aflatoxins on food products of 15 and 20 ppb (ppb = 1 mg per metric ton = ng/g) (30). The Food and Drug Administration imposes a limit of 20 ppb for interstate commerce of food and feed, and a limit of 0.5 ppb of aflatoxin $M_1$ for sale of milk (10). European countries have standards of 4 ppb and other nations such as India have limits of 30 ppb. In the United States, the standard for animal feed is 300 ppb (77). Because of this, aflatoxin accumulation can cause significant economic losses but in general aflatoxins do not reach the food chain. It has been estimated that maize producers in North Carolina lost $97 million in 1980 due to aflatoxin contamination (47).

Unfortunately, not all countries have effective regulations for levels of aflatoxins in food products. In many developing countries, maize is primarily used for human food, and even if there were specific standards for aflatoxin contents and a way to determine them, the presence of toxic compounds may not be enough to prevent people from using that food (39, 77). Some of the poorest people in the world are subsistence farmers who consume the maize that they produce. This could be dangerous because it has been proved that presence of aflatoxins in the diet increases the risk of liver cancer 3.3 fold (68), impairs child growth and development (24), and interferes with the immune system (30, 39).

In people exposed to the hepatitis B virus, aflatoxin induced risk of liver cancer increases significantly (26, 77). The United Nations organization that develops food standards, Codex Alimentarius, does not specify recommended aflatoxin limits because of fears that this might force developing countries to retain contaminated products for local consumption. As a result, more emphasis has been placed in vaccination for hepatitis control as a means to reduce hepatic cancer rather than in regulating levels of aflatoxins (30). Codex Alimetarius also recommends cultural practices to reduce aflatoxin $B_1$ in raw materials, including the use of varieties resistant to *A. flavus*.

***Life Cycle***

*A. flavus* inhabits the soil, where it decomposes plant and animal material (62). Sclerotia can serve as an overwintering structure, but it has also been shown that *A. flavus* can overwinter as mycelium and conidia (47). Low background populations of *A. flavus* have been reported in soils and atmosphere, but deposits of infested waste

maize were found to be the source of primary inoculum in field experiments (50). Conidia are the primary inoculum. Injury caused by insects increases the chances of infection, but is not indispensable for disease (62).

In addition to ear rot in maize, *A. flavus* causes damping-off and yellow mold in peanut (*Arachis hypogea* L.) as well as lint contamination in cotton (*Gossypium* spp. L). It is also found in insect frass, and it colonizes dead and parasitized insects. It causes Stonebrood disease of honey bee (*Apis mellifera* L.) and Koji Cabi disease of silkworm (*Bombyx mori* L.) (18). *A. flavus* is capable of infecting animals and causes symptoms ranging from hypersensitivity to invasive pulmonary infections in humans (29).

In maize, cytological studies have shown that the fungus typically colonizes the silk at the yellow-brown stage and then the glume tissue in the maize ear. Maize silk is the main entry point into the ear and the kernels in the absence of physical damage. Although there are substantial amounts of dead silk tissue at this time, *A. flavus* has been reported to kill the silk ahead of its invasion in a necrotrophic interaction (66). Systemic infection of maize plants though the stalk is extremely limited (86). On its way to the maize kernels, mycelium has been observed to colonize spikelets through the junction of the bracts and rachillas or through the air space between the rachis and spikelets. Then the fungus penetrates into the grain through the upper rachilla (66). Recently, another ear rot pathogen of maize, *Fusarium verticillioides*, was found to penetrate the maize kernel through the stylar canal (19). In the kernel, *A. flavus* colonizes the aleurone layer and forms a fungal mat

between the germ and the endosperm (G.A. Payne, *personal communication*). No aflatoxins have been found independent of *A. flavus* hyphae in inoculated maize ears (65). The distribution of aflatoxin levels in an infected maize ear varies widely even for grains that are next to each other (65).

### ***Aspergillus flavus* Populations**

Populations of *A. flavus* are highly polymorphic and produce variable amounts of aflatoxins (4). The quantitative genetics of aflatoxin synthesis have not, however, been characterized. Morphologically, depending on the size of the sclerotia, *A. flavus* has been divided into two types: S strains that produce small and abundant sclerotia, and L strains that produce large sclerotia. In addition, S strains in general produce more aflatoxins than L strains (5). Limited sampling in Kenya also has suggested that S strains are more often associated with outbreaks of aflatoxin contamination than L strains (58). In a study of soil populations in the USA, *A. flavus* was found to be the dominant *Aspergillus* species in soil samples. Significantly more *A. flavus* was found in Central Texas, Georgia and Alabama than in Virginia, North Carolina and western Texas (32). In general, the frequency of soil samples containing *A. flavus* and *A. parasiticus* increase from subtropical to tropical latitudes (32).

In addition to these strain types, vegetative compatibility groups (VCGs) biologically divide *A. flavus* into an unknown number of groups (62). VCGs divide isolates based on their ability to form heterokaryons. Recently, using population genetics analyses, it was found that VCGs are sexually isolated with no recombination

even between groups from different mating types (27). Sexual reproduction of both *A. parasiticus* and *A. flavus* has been observed under laboratory conditions after leaving plates with opposite mating types at 30°C for 6 to 11 months (33). The teleomorphs were named *Petromyces parasiticus* and *P. flavus*, respectively, because of the morphological similarities to *P. alliaceus*, a non-aflatoxigenic species (33). It is not known whether *A. flavus* reproduces sexually in more tropical latitudes where high temperatures in the soil are common.

Interestingly, non-aflatoxigenic *A. flavus* strains have been shown to reduce aflatoxin contamination (9) to the extent that atoxigenic strains are now being used as biological control agents in several crops. The mechanisms by which aflatoxin accumulation is reduced by the non-aflatoxigenic strain are not clear, especially since competitive exclusion has been shown not to explain all the effect (43).

***Management***

Pre-harvest aflatoxin contamination is mainly managed by cultural practices, which have a limited effect. Practices that lead to healthy plants are usually recommended, such as avoidance of drought stress by planting at appropriate times or irrigation and deep tillage (49). Insect control reduces aflatoxin concentrations but is not necessarily cost-effective (10). *A. flavus* does not require insect presence to infect maize ears, because it can enter through the silk channel. Studies have been conducted on the effect of genetically modified maize lines that are insect resistant (Bt-corn) on mycotoxin accumulation. Despite some contradictory results (49, 54),

the overall trend in carefully-designed experiments indicates that Bt hybrids can reduce the levels of aflatoxin when insects are present (82). The reduction of aflatoxin levels in these studies was not below the threshold set for human consumption in the US.

Another important pre-harvest practice is the use of resistant hybrids or varieties. Moderate levels of resistance to aflatoxin accumulation have been incorporated into some hybrids. Resistance by itself, however, may not be sufficient to prevent high concentrations of aflatoxin (49, 73). The genetics of resistance to *A. flavus* are reviewed and discussed below.

Post-harvest accumulation of aflatoxins can be avoided through the use of proper storage conditions (e.g. drying kernels to 15% or less within 24 to 48 hours of harvest) (49, 69). Resistance in mature kernels has been reported (11, 12) and it might be different from pre-harvest resistance (S. Mutiga and R. Nelson, *unpublished*). After contamination has occurred, intervention is still possible to reduce damage to human populations. Some possibilities include the use of trapping agents such as NovaSil or detoxifiers such as chlorophyllin (26). NovaSil is a naturally-occurring clay which selectively binds aflatoxins, producing no side effects to humans (1). Clorophyllin, derived from chlorophylls sequesters aflatoxins, also without adverse effects to humans (20). There is also secondary intervention systems that reduce the risk of liver cancer such as green tea polyphenols and others reviewed by Groopman et al. (26).

***Drivers of Pre-Harvest Epidemic Severity in Maize***

Although *A. flavus* populations are found in soils through the year, epidemic severity varies widely (18). Several factors that affect the pathogen, the host, the environment and their interactions over a year cycle are responsible for this variation. For example, a recurrent theme in the literature is the effect of drought stress in aflatoxin accumulation. As indicated previously, more *A. flavus* is found at lower latitudes. The risk of aflatoxin contamination is greatest between 35°N and 35°S. However, whether it is at low or high latitudes, aflatoxin contamination can be perennial, sporadic or infrequent depending on the specific location (18). Several studies (e.g. (87) describe strong year-to-year variation in the levels of contamination at the same location. Furthermore, infection levels of the crop are also extremely variable. For instance, two kernels located next to each other on the same ear were reported to have aflatoxin levels of 0 and more than 15 ppb (18), but the real range in natural infection surely exceeds that. The specific source of variability in multiple-year studies has been studied to some extent but is not understood.

On the pathogen side, there is extensive genetic diversity among populations of *A. flavus*. This is reflected in variation in mycotoxin production, morphology, genetic fingerprints and the large number of vegetative compatibility groups (VCG). In addition the amount of natural inoculum is another variable. It has already been stated that *A. flavus* is more common in tropical soils. Populations of *A. flavus* vary significantly from season to season and from year to year (18), presumably due to

environmental changes. Given that inoculation techniques that challenge the plant with high numbers of spores and with controlled wounding produce more consistent results (87) it may be the case that inoculum levels are an important driver of natural epidemics.

Environmental effects such as rainfall are an important factor for aflatoxin accumulation in cotton seed (17). In maize, however, temperature and not rainfall was correlated with aflatoxin accumulation (87). Greenhouse studies have also implicated high temperature as a main driver of *A. flavus* colonization and aflatoxin accumulation in maize (53). Further analyses under natural conditions are complicated due to the sporadic occurrence of the disease without inoculation. However, the effect of the environment on aflatoxin accumulation and *A. flavus* populations should be further studied.

With regard to the maize host, natural variation in maize lines for aflatoxin accumulation exists (6, 7, 10, 84). Early studies indicated that aflatoxin accumulation starts long before harvest (52, 53). Significant differences in the levels of aflatoxin on resistant versus susceptible lines were found 60 days after female flowering (85). This resistance to aflatoxin accumulation could be due to several factors. Lower levels of aflatoxin can be correlated with traits such as flowering time and fiber content in kernels (Chapter 3). Some studies have reported significant genotypic and phenotypic correlations between aflatoxin accumulation and (among other traits) endosperm texture (7). Conversely, no correlation was reported in one study among aflatoxin

content and endosperm texture or kernel content traits estimated by near-infrared spectroscopy (3).

Finally, the interaction of host and environment is confusing. Moreno and Kang (47) review several reports of plant stress significantly increasing the levels of aflatoxin. These factors are drought, nutrient deficiencies, and insect and weed infestations (47). The effects of environment on the host suggest that the maize plant may be using active defense mechanisms against *A. flavus* under normal conditions, that the plant is incapable of maintaining under stress, resulting in higher levels of disease. Another posibility is that stress on plants could have developmental or structural consequences, such as slower closure of the physical pathways that the fungus uses to gain access into the kernel.

***Resistance***

Plants are resistant to most fungi though mechanisms that range from avoidance mechanisms by which pathogens are kept at a safe distance by morphological features, to an innate immune system that recognizes pathogen compounds and triggers reactions that stop the intruder. Researchers have reported resistance to aflatoxin accumulation and *A. flavus* infection since the early 1970s (49). However, the challenge posed by this pathosystem, especially the genotype by environment interaction, has been such that only low levels of resistance are available in elite lines, and the mechanisms of resistance to *A. flavus* remain unknown. It is important to note that resistant materials could have orders of magnitude lower levels

of aflatoxin than susceptible genotypes, indicating that resistance could be a useful tool. For example, a resistant line accumulated 38 ng of aflatoxin per gram after a strong pathogenic challenge while a susceptible control reached 3710 ng of aflatoxin per gram (84). Other challenges for the proper understanding and deployment of resistance were to characterize the disease cycle, after which effective inoculation techniques had to be developed (10). A fundamental difficulty is the low levels at which aflatoxins are dangerous. A key limitation for this area of research, and for surveillance and management in the food system, is that expensive tests need to be conducted for accurate measurements. These problems remain but much has been learned and sources of resistance are now available.

Several maize breeding programs located in aflatoxin-prone areas of the USA have developed and released maize lines that are resistant to aflatoxin accumulation. Mp420 and Mp313E were developed and released in the early 1990s in Mississippi (63, 64). More recently the same program has developed and released Mp715 and Mp717 (79, 80). The GT-Mas:gk population was described as resistant to *A. flavus* (10). Screening of inbred lines in the midwest US showed that MI82, CI2, T115, Tex6, LB31, CI2 and Oh513 are also resistant to aflatoxin accumulation (10). In addition, inbred lines NC400, NC408, NC388, and CML348, as well as two accessions from the Germplasm Enhancement of Maize project (GEM), were found to be resistant in two locations (84).

More development of resistant materials comes from research institutes with international mandate. Scientists at the International Institute of Tropical Agriculture, in collaboration with the United States Department of Agriculture, have developed inbred lines resistant to *A. flavus* in Nigeria. Six lines that were resistant in *in-vitro* inoculation assays and that accumulated lower levels of aflatoxin in field assays have been released (45). Tropical lines have also been evaluated elsewhere; Betrán et al. (6) reported that CML269 and CML322 as well as Tx772, CML285, CML326 and FR2128 are good sources of resistance for white and yellow endosperm inbreds respectively. CML322 was highlighted because of respectable yield and resistance to insects (6). QTL positions on the maize genome for a cross between CML322 and B73 are reported in Chapter 4. Another source of resistance is CIMMYT's population 69 of flinty orange germplasm (7).

As indicated earlier, the mechanisms by which resistant maize lines accumulate lower levels of aflatoxin are unknown. In addition to aflatoxin accumulation, several traits related to *A. flavus* pathogenesis have been evaluated. *In-vitro* screening of maize inbreds for colonization using a GUS transformed strain resulted in the finding that some lines that were resistant to *A. flavus* still had high levels of aflatoxin accumulation (12). This indicated that related components of resistance might not always be correlated. On the other hand, the expense and difficulty of evaluating multiple breeding lines for mycotoxin levels prompted investigators to rate other traits with the hope that they would be correlated with aflatoxin accumulation. Using multiple inoculation techniques, various components

have been evaluated through the years and it is important to clearly distinguish them, as they might not always be correlated (12). Examples of other components of resistance to *A. flavus* are ear rot severity, kernel sporulation, and bright greenish yellow fluorescence (BGYF, a sign of kojic acid, another toxin produced by *A. flavus*). In addition, at least two different maize tissues are involved in the infection process: silk and kernels. Some authors have also looked for resistance in the cob (55); the stalk has been proven to play an extremely limited role (86).

Silk resistance presents an interesting case because there are several lines of evidence that the maize silk is capable of restricting the growth of *A. flavus*. First, there is clear evidence of silk resistance to *Fusarium graminearum* (another ear rot pathogen) in the line Co272 (59). Second, *A. flavus*-susceptible hybrids had the same levels of contamination when directly inoculated via kernels or silk, while more resistant hybrids had differences depending on the inoculation method (87), suggesting that silk plays a role in resistance. Finally, chitinases and other proteins have been found to differentially accumulate in silk tissues of lines that are resistant or susceptible to *A. flavus* (56).

***Genetic Basis of Resistance***

The genetic basis of resistance to aflatoxin-related traits has been studied using diallel crosses and QTL mapping populations. Early studies showed the absence of complete resistance and suggested a strong importance for general combining ability, which suggests that the genetic effects are mainly additive. In some reports, however,

specific combining ability was the main effect, indicating the presence of dominant or epistatic gene effects (6, 13, 25, 81). More recent QTL mapping experiments confirm the importance of additive genetic effects and indicate the existence of at least 14 regions of the genome where QTL from multiple studies co-localize. Most of these QTL have small additive effects (71, 72).

Other traits that have been studied in relation to *A. flavus* resistance are BGYF and ear rot severity. Pearson correlations between BGYF and aflatoxin accumulation have been reported between non-significant 0.21 and a significant 0.67 (13). BGYF expression by *A. flavus* strains seems to vary depending on the source of resistance; as a result, this trait was not recommended for selection of resistant lines (13). The relationship between ear rot severity and aflatoxin accumulation has also been studied. The correlation over two years varied from a significant 0.53 to no correlation in the second year in $BCS_1$ families of B73 x Oh516 (13), or it was stably significant between 0.41 to 0.64 in two years for $BCP_1S_1$ families from B73 x MI82 (42). The possibility of using alternate traits for aflatoxin accumulation such as grain composition or plant traits such as husk coverage have been suggested (25). Two studies have found significant correlations between aflatoxin accumulation and other traits: grain texture, husk cover, grain yield and silk channel length (3, 6).

Development of real-time PCR methods for the estimation of fungal biomass now allows adding this component to the studies of resistance. We have reported a strong correlation (0.85) between aflatoxin accumulation and colonization estimated

by qPCR (46). Colonization estimated by qPCR has also been studied in a complete diallel cross. Significant correlations were found between fungal biomass and aflatoxin (0.90) and ear rot ratings (0.51). General combining ability and specific combining ability were, however, not significant for fungal biomass. For ear rot and aflatoxin accumulation, general and specific combining abilities were significant and as usual, general combining ability was a larger source of variance than specific combining ability (83), suggesting that additive genetic effects are the norm.

The inheritance of resistance to *A. flavus* has been studied in at least seven maize populations (Table 1). Broad sense heritabilities ($H^2$) to aflatoxin accumulation ranged from non-significant (0%) to 74%. In most of the studies, the heritability of resistance to aflatoxin accumulation was significant and greater than 50%. Ear rot heritabilities ranged from non-significant (0%) to 66%. Heritabilities for multiple traits were rather high for some populations such as B73 x M182 (42). This could be due to the environmental conditions on which this population was tested or other factors. Contrastingly, there was no $H^2$ in the B73 x Tex6 population for ear rot, and for aflatoxin the $H^2$ was low (51). Overall, low to intermediate levels of heritability are found for aflatoxin accumulation and ear rot in multiple populations indicating that gains in resistance should be achievable.

Table 1. Estimates of broad sense heritability ($H^2$) and narrow sense heritability ($h^2$) for aflatoxin resistance and other related traits reported in the literature.

| Study | Population | Trait | $H^2$ | $h^2$ |
|---|---|---|---|---|
| | | | ---- % ---- | |
| Hamblin and White (28) | (B73/Tex6)$F_3$ | Aflatoxin | 63 | 45 |
| | | Ear rot | 58 | 39 |
| | (Mo17/Tex6)$F_3$ | Aflatoxin | 65 | |
| | | Ear rot | 66 | |
| Walker and White (70) | (B73/CI2)$F_3$ | Aflatoxin | 32 | 25 |
| | | Ear rot | 48 | 39 |
| | ($B73^2$/CI2)$S_1$ | Aflatoxin | 26 | 17 |
| | | Ear rot | 37 | 25 |
| Maupin et al. (42) | ($B73^2$/M182)$S_1$ | Aflatoxin | 74 | |
| | | BGYF | 84 | |
| | | Ear rot | 63 | |
| Paul et al. (51) | ($Tex6^2$/B73)$S_1$ | Aflatoxin | 19 | |
| | | Ear rot | ns. | |
| Busboom and White (13) | ($B73^2$/Oh516)$S_1$ | Aflatoxin | ns. | |
| | | BGYF | 21 | |
| | | Ear rot | 11 | |
| Mideros et al. (Chapter 4) | (B73/CML322)$S_5$ | Aflatoxin | 63 | |
| | | Colonization | 11 | |
| | | Sporulation | 14 | |

***Mapping QTL for Aflatoxin-Related Traits***

The first reported mapping experiment for resistance to *A. flavus* was conducted in the cross GT-A1 x GT119 (74). GT-A1 is an inbred developed from the GT-MAS:gk population. Although the authors found QTL for silk maysin, husk tightness and husk phenotype, no markers associated with resistance to aflatoxin were reported (74). Paul et al. (51) conducted a QTL study with two populations of the cross Tex6 x B73. Several QTL were found in this study by multiple regression analysis in two years and by composite interval mapping in one year. Some regions

that were present in more than one analysis were in chromosomal bins 4.07 and 4.08 (51). In addition to a complete characterization of the genetics of resistance to aflatoxin concentration in a cross of Oh516 by B73, Busboom and White (13) mapped QTL for resistance to ear rot, BGYF and aflatoxin accumulation. QTLs for resistance to aflatoxin were found on chromosomes 2, 3 and 7.

A series of $F_{2:3}$ mapping populations have been studied in Mississippi. Brooks et al. (8) mapped QTL for resistance to aflatoxin accumulation from Mp313E x B73. Two QTL were found in three out of four environments: one on chromosome 2 and another on chromosome 4. Warburton et al. (72) mapped QTL in the Mp717 x NC300 population, finding QTLs on all but chromosomes 4, 6 and 9. QTL on chromosome 7 were observed in two years (72). Finally, Warburton et al. (71) found QTL in multiple years on chromosomes 1, 3, 5 and 10 using the Mp717 x T173 population.

Robertson-Hoyt et al. (61) used a subset of recombinant inbred lines (RILs) from the population NC300 x B104 to analyze the relationship between resistance to aflatoxin and fumonisin. Twenty-four lines with the highest resistance and susceptibility to fumonisin accumulation were used for mapping resistance to aflatoxin and fumonisin accumulation as well as for ear rot. QTLs on chromosomes 5 and 8 had effects on both mycotoxin traits. One QTL on chromosome 3 affected both ear rot traits. These results added support to the correlation of resistance between the two ear rots (61).

### *Maize Proteins and Genes Involved in Resistance*

Several studies have been conducted to identify proteins involved in resistance to *A. flavus* or to the accumulation of aflatoxins; these were reviewed by Luo et al. (40). Some examples include ribosome-inactivating proteins, trypsin inhibitors, zeamatin (15), pathogenesis-related protein 10 (PR10) (16), catalase (41), and oxylipins (21). PR10 was silenced using RNAi to confirm its involvement in resistance to aflatoxin accumulation (14). Catalase3 of maize was found to have a higher activity in a resistant line when compared to a susceptible one and sequencing comparison pointed to a 20-amino acid deletion in the former (41). Oxylipins, which are part of the jasmonic acid pathway and are involved in plant signaling, have also been shown to be involved in resistance to *A. flavus*. Surprisingly, disruption of *ZmLOX3* (an oxylipin) results in plants that are susceptible to *A. flavus* while these same plants are resistant to other maize ear rot pathogens (21). No resistance genes have been cloned for resistance to *A. flavus* using a map-based approach but resistance to Gibberella ear rot was associated with a guanylyl cyclase (89) by positional mapping.

Proteomics have also been used to compare silks of resistant and susceptible maize lines. Different levels of accumulation were found for several silk proteins when resistant vs. susceptible inbreds were compared. Among the differentially-expressed proteins were several antioxidant enzymes, PR10, chitinases, and germin like proteins (56). Chitinase activity assays indicated that the resistant inbreds degraded chitin better than susceptible ones (57).

Kelley et al. (36) used microarrays to analyze gene expression in resistant and susceptible lines. They report over 200 genes whose expression patterns changed after challenge with *A. flavus* (36). No reliable pattern was distinguishable from the study and these microarray results still require confirmation. However, this study clearly indicated that the maize plant undergoes considerable changes in transcription levels as a result of *A. flavus* infection and that pathways involved in transport, protein modification and metabolism are reprogrammed in the plant. A dedicated database is available online that permits searches of this expression data (37). In a separate study, four genes from this data set were tested for expression by qRT-PCR. Transcripts for a predicted transposon increased significantly two days after inoculation in the resistant line. Transcripts for a predicted auxin-responsive gene and for an indole-3-glycerol phosphate lyase increased significantly three days after inoculation in the resistant line, while a protein involved in ethylene signaling increased in both the resistant and susceptible lines, although to a lesser extent in the latter (2).

Analysis of the cob proteome of resistant (Mp313E and Mp420) and susceptible (B73 and SC212m) inbreds suggested that tissue from the resistant lines had more constitutive defense proteins while that from susceptible lines had more induced defense proteins (55). Forty-two proteins were identified as more abundant in resistant inbreds while 26 were more abundant in susceptible inbreds. In addition, significant differences were found over time, suggesting that resistant lines accumulate constitutive defenses faster than susceptible lines. This study highlights several

candidate genes, many of which had been located in previously described QTL regions (55).

***Conclusions and Future Work***

Awareness of aflatoxin contamination in food systems has increased in the last five years, since work related to this dissertation began. Because of the complexity of the problem, solutions could derive from diverse fields including human health, nutrition, policy and agriculture, among others. It is clear that resistance to aflatoxin accumulation exists in maize. However, there is a substantial lack of knowledge about what this resistance is and if it could be effective as management method. Interventions to reduce the levels of aflatoxin contamination could include the development and deployment of plant varieties that accumulate lower levels of aflatoxin. We work under the reasonable assumption that the use of resistant varieties would significantly reduce the levels of aflatoxin that reach food chains in developing countries.

In this dissertation, after a phenotypic dissection of the trait in Chapter 3, we find that resistance to aflatoxin accumulation is strongly correlated with resistance to colonization of *A. flavus* in kernels. We also find that there is natural variation in maize lines for resistance to colonization in silks and kernels. These results suggest that resistance may be due to plant defense mechanisms that slow the colonization in maize. However, there is also evidence for other mechanisms to be involved in resistance. A significant correlation between flowering time and lower aflatoxin

levels suggests that avoidance might be another important part of resistance. Resistant inbred lines tend to flower later in the season arguably experiencing the pathogen challenge under environmental conditions that are more favorable to the host. Surprisingly, kernel morphological traits at maturity were also correlated with aflatoxin accumulation. This suggests the hypothesis that structural features in the kernel might prevent pathogen ingress. Microscopic analysis of these structures might reveal new entry points such as those recently described for *Fusarium verticillioides* (19).

Further phenotypic characterization of resistance mechanisms is complicated by the significant year-to-year variation. After carefully controlling the environment with *in-vitro* inoculation procedures in Chapter 3, we conclude that this variation must be due to the environment in which the plant is grown, and that this environmental effect on the mother plant significantly changes the expression of resistance in the silk and kernels. A testable hypothesis is that weather has a significant effect on kernel defense capabilities. Some alternatives for exploration in this area are the accumulation of preformed defense substances, structural variation and endophyte colonization of kernels under diverse weather conditions.

The genotypic dissection of resistance in Chapter 4 confirms that there is moderate heritability for this trait and that selection is possible for resistance to aflatoxin accumulation. At least one QTL on chromosome 4 was confirmed using near isogenic lines. The importance of chromosome 4 as a source of resistance is also

highlighted in Chapter 5. Even after reducing the confidence intervals with the meta-analysis of QTL, the smallest region of the maize genome associated with aflatoxin accumulation is 1Mb (mqcAFL4.09). This region contains a couple dozen predicted genes in the reference maize genome. None of these genes is one of the proteins previously implicated in resistance. Although these results are encouraging confirmation of the metaqtl should be conducted using break point analysis in a by-parental population, or association mapping in a panel of diverse maize lines, or with near-isogenic lines. In the future, positional mapping could be used in the maize genome regions that harbor promising QTLs (for example those that are effective against multiple ear rot pathogens). Proteins that have been implicated in resistance to aflatoxin accumulation could be used as candidate genes.

The ultimate goal of this dissertation is to facilitate the incorporation of resistance into maize. The metaQTL analysis in Chapter 5 significantly reduced the confidence intervals of QTL for resistance to multiple components of resistance and one marker was validated with one set of near isogenic lines in Chapter 4. Marker-assisted selection requires tightly linked and validated molecular markers (67). Considering that most of the QTL for resistance to aflatoxin accumulation have limited phenotypic effect, multiple QTL should be introgressed into elite lines. Thus, more markers need to be validated. Crop improvement, in the case of aflatoxin resistance, should be applied to populations using methods such as marker-assisted recurrent selection (67). Another alternative is the use of genomic selection, which is

well positioned to incorporate multiple QTL with small effect in a trait that has moderate levels of heritability (34).

Finally, it is important to point out that very little is known about pathogen populations, especially in the areas of the world where the disease is most prevalent. Proper deployment of any form of resistance requires a good understanding of the actual populations in the field and of the effect that resistance could have on them (67).

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# CHAPTER 2

## *Aspergillus flavus* BIOMASS ESTIMATION BY QUANTITATIVE REAL-TIME PCR[1]

### *Introduction*

*Aspergillus flavus* Link:Fr is a widely distributed fungus that spends most of its life cycle as a saprophyte in the soil (30). It is also an opportunistic pathogen that is able, under certain conditions, to cause disease in plants and animals, including maize (*Zea mays* L. subsp. *mays*) and humans (8, 13). *A. flavus* is a common cause of ear rot of maize in warm climates. This fungus can also contaminate a number of agricultural commodities with a wide array of secondary metabolites, some of which are toxic to humans and farm animals. Of particular relevance are aflatoxins produced by some strains of *A. flavus*, *A. parasiticus*, and *A. nominus*. Aflatoxins are polyketides that often accumulate in infected plant seeds such as maize kernels or peanuts (30). The B1 form of aflatoxin is the most potent carcinogen found in nature. While moderate exposure leads to cancer in humans, acute aflatoxicosis causes direct liver damage that often results in cirrhosis (15, 40). Perhaps more important and not as widely reported are the effects of chronic exposure, which cause immunosuppression and nutritional interference (39).

[1] Mideros, S. X., Windham, G. L., Williams, W. P., and Nelson, R. J. 2009. *Aspergillus flavus* biomass in maize estimated by quantitative real-time PCR is strongly correlated with aflatoxin concentration. Plant Disease 93:1163-1170

The strict regulation on trade of contaminated maize leads to economic burdens on farmers in developed countries. In developing countries, where regulations may be nonexistent or not enforced, and where consumption of home-grown maize is typical, people may be widely exposed to this toxin. High concentrations of aflatoxins are consumed by humans in areas of the world that have higher than average levels of liver cancer, childhood malnutrition, and disease. Many of these health problems interact with and are exacerbated by aflatoxicosis, increasing morbidity or mortality (33). For example, aflatoxin and the hepatitis B virus have synergistic effects in causing liver cancer (15, 40).

*A. flavus* conidia are the primary source of inoculum and infect developing maize ears. Cytological studies indicate that the silk tissue after pollination is the primary portal of entry into the maize ear (22). Mycelium then colonizes the young kernels through the rachilla. It has been observed that the pathogen destroys the cells ahead of itself in a typical necrotrophic interaction (34). High temperatures (>30°C) and drought favor the development of this disease (30).

Control measures generally consist of pre-harvest cultural practices that reduce plant stress, peri-harvest practices that reduce grain moisture, and post-harvest management practices that maintain low grain humidity and avoid pest infestation. However, cultural practices have a limited effect, especially with pre-harvest aflatoxin contamination. The development of pre-harvest host resistance to aflatoxin

contamination is an important component of integrated management (25). Progress on this strategy has been limited however, due in part to the complexity of this trait. Aflatoxin resistance behaves as a quantitative trait, which presents relatively low levels of heritability and extremely high environmental effects. Maize lines with high degrees of resistance have been identified and low-resolution QTL maps have been generated for reduced aflatoxin accumulation and Aspergillus ear rot resistance (2, 6, 27, 38). These forms of resistance have not been consistently incorporated into elite maize lines.

Resistance to *A. flavus* kernel infection and accumulation of aflatoxin may be seen as distinct traits (24, 46). Two reports examining the relationship between the two traits suggested strong but variable correlations (29, 36), while a third study indicated that the two traits were independent (5). An African inbred found to be resistant to aflatoxin production allowed high levels of *A. flavus* growth, as measured using a GUS-transformed strain. In addition, certain inbreds that were found to be susceptible to aflatoxin accumulation supported low levels of fungal growth (5). There is also indirect evidence from QTL mapping experiments in which loci affecting ear rot and aflatoxin accumulation were associated with different molecular markers, suggesting that the loci mapped to distinct chromosomal regions (6). Further support for the separate nature of fungal growth and aflatoxin accumulation was inferred from the effect of antioxidant compounds such as caffeic acid on *A. flavus* in culture, which reduced aflatoxin content more than 95% while fungal weight on membrane filters was unaltered. Microarray analysis indicated that genes in the biosynthetic pathway of

aflatoxins were significantly down-regulated in the presence of caffeic acid (19). It has been recently proposed that aflatoxigenesis is a fungal reaction to oxidative stress (18, 19). It is possible that maize lines that produce more reactive oxygen species (ROS) in response to *Aspergillus* infection accumulate higher concentrations of aflatoxins.

Further exploration of this and related hypotheses requires sensitive tools for the measurement of both aflatoxin and *A. flavus*. ELISA assays are available for the measurement of aflatoxin, but tools are needed that allow efficient and specific measurement of fungal biomass. Conventional assessment methods for Aspergillus ear rot do not provide accurate evaluations of the levels of infection, because they only allow rating of the superficial signs of the fungus. Percentage ear rot has the disadvantage of subjectivity, which adds error to the already environmentally-affected trait. Accurate measurements can be achieved using transformed strains of *A. flavus* that express either GUS or GFP (3, 11, 29), but the use of transgenic strains in the field requires compliance with regulatory guidelines and limits the range of strains that may be utilized. For these reasons, we developed a quantitative real-time PCR (qPCR) technique for the evaluation of infection levels in maize kernels.

qPCR is a modification of the traditional PCR that measures the amount of amplification product at every cycle of the reaction. Two different florescent dyes can be used to measure the increase of PCR product. The SYBR green dye binds to double stranded DNA and the fluorescence increases as a result of the logarithmic

growth of the target sequence. The other method is the use of TAQMAN sequence-specific probes with dual fluorochromes (1) (14). One of these labels is a reporter (such as VIC or FAM) and the other is a quencher (TAMRA). Due to physical proximity of the reporter to the quencher, the probe is not fluorescent until the polymerase separates the two labels during each of the amplification cycles. The fluorescence due to SYBR green or the reporter dyes in the TAQMAN reactions is measured after every replication cycle of the PCR. When this fluorescence exceeds a specific threshold, a Ct value is produced. This Ct value is compared to a standard curve of known quantities of DNA and the concentration of DNA in the unknown sample can be inferred (1, 14). Pathogen DNA concentration in a sample of host tissue can be used as an estimator of fungal biomass (1, 28). Recently, a TAQMAN qPCR technique was used to quantify *A. flavus* in pure culture but its use in the presence of DNA from other species including corn in the same sample was not validated (9).

In this paper we report the development and validation of two quantitative real time PCR (qPCR) techniques for the accurate estimation of fungal colonization in maize grain. One important application of this assay is in characterizing the nature of aflatoxin in maize germplasm. In particular, it is of considerable practical and biological interest to determine whether there are maize genotypes that reduce the levels of colonization of *A. flavus* without triggering the accumulation of high aflatoxin concentrations, and conversely whether maize genotypes exist that suppress fungal growth while inducing toxin accumulation. Because there is evidence for the

induction of aflatoxin production by reactive oxygen species and a reduction in the toxin concentration caused by certain antioxidants (17), we tested the hypothesis that different types of *A. flavus* resistance have differential effects on fungal biomass and aflatoxin accumulation. For this purpose, we used the TAQMAN method developed in the first part of the study to analyze both aflatoxin concentrations and fungal DNA concentrations on hybrids that were field-inoculated in Mississippi. The parents of these hybrids contain early tropical and non-stiff stalk maize lines as defined by a genetic diversity study by Liu et al (2003) and are maintained by the USDA-ARS Corn Host Plant Resistance Research Unit at Mississippi State University. Because the strong correlation found on these hybrids could have been attributed to common source(s) of resistance, we subsequently tested a set of 18 diverse inbred lines. These inbreds are a subset of the founders of the "nested association mapping" population that have been developed to maximize the number of alleles captured for quantitative genetics studies (45). These genotypes contained not only tropical and non-stiff stalk maize lines but also stiff stalk lines and lines with mixed ancestry (12, 21).

### *Materials and Methods*

**Primers and TAQMAN Probes.** Three pairs of *A. flavus*-specific primers and their respective probes were designed in the internal transcribed spacer 1 (ITS1) using Primer Express 1.5 (Applied Biosystems, Foster City, CA). Sequences of the ITS1 regions were obtained from GenBank for *A. flavus* (AB000532), and the closely related species *A. oryzae* (AB00533), *A. sojae* (D84357), *A. parasiticus* (D84356), *A. tamarii* (D84358) and *Emericella nidulans* (AB243115) (30, 37). The sequences were

aligned with ClustalX 1.81 (35). Primers were designed to correspond to regions with the highest polymorphism between species as indicated by ClustalX. Primer pair Af2 (forward primer: 5'-ATCATTACCGAGTGTAGGGTTCCT-3'; reverse primer: 5'-GCCGAAGCAACTAAGGTACAGTAAA-3') was used for the SYBR green and TAQMAN reactions, resulting in an amplified product of 73 bp. The Af2 TAQMAN probe was: (5'FAM-CGAGCCCAACCTCCCACCCG-3'TAMRA).

For maize, four pairs of primers were designed using the maize alpha tubulin sequence obtained from GenBank (x73980.1). Primer pair Zmt3 (forward primer: 5'-TCCTGCTCGACAATGAGGC-3'; reverse primer: 5'-TTGGGCGCTCAATGTCAA-3') was used for the SYBR green reactions, resulting in an amplified product of 63bp. In addition to the primers designed for this experiment, the primers INCW2-97 designed by Murray et al. (26) were tested in the optimization assays for the TAQMAN reactions.

**Quantitative PCR Development and Optimization.** Two qPCR methods, SYBR Green and TAQMAN, were developed and validated. All the qPCR experiments were conducted in an Applied Biosystems ABI PRISM 7000 Sequence Detection System, with 96 well reaction plates and optical adhesive covers or optical caps (Applied Biosystems, Foster City, CA). Sequence detection primers and TAQMAN TAMRA probes were also obtained from Applied Biosystems.

*SYBR Green Optimization.* The specificity of each primer and the optimal annealing temperature were determined by gradient PCR with control DNA of *Fusarium graminearum* and *Penicillium sp.* because these fungi are commonly found in maize ears. Reactions for optimization were conducted as recommended for Power SYBR Green PCR Master Mix (Applied Biosystems). qPCR reactions were first tested with a profile of 95°C for 10 min and 40 cycles of 95°C for 30s, 37°C for 30s and 72°C for 30s. DNA samples were pure pathogen or host DNA at 10 ng/µl and a mixed sample of 10 ng/µl of pathogen diluted in 1 ng/µl of host DNA. Each reaction was prepared in 25 µl with 1X Master Mix, 3 µl of template and variable concentrations of forward and reverse primers. Af2 and Zmt3 primer concentrations were tested at 200, 75 and 50 nM. A dissociation curve was created for each reaction. Temperature profiles were adjusted to reduce the presence of dimer. A two-step profile was tested and reduced times for each step of the cycle were also assessed.

To create standard curves, pathogen DNA was diluted in water at 10, 1, 0.1, 0.01, 0.001 ng/µl. Host DNA was diluted to 100, 10, 1, 0.1, 0.01, 0.001 ng/µl and a mixed DNA standard curve was prepared by diluting pathogen DNA at 10, 1, 0.1, 0.01, 0.001 ng/µl in 1 ng/µl of host DNA. Aliquots were prepared and frozen at -20°C, keeping a working dilution at 4°C. For the efficiency calculation of each primer pair, qPCR was conducted in 25 µl reactions and 40 cycles. Efficiency of the reaction was determined by the formula: $E = 10^{(-1/slope)}-1$, as recommended by the Applied Biosystems real-time PCR training modules. When using a standard curve with a logarithmic dilution, such as the one used in this study, a 100% efficient reaction would have a slope of -3.3386.

This efficiency value can be used to evaluate the quality of the primer design for absolute quantification of DNA in a qPCR reaction. Slopes between 90-110% efficiency are generally considered acceptable.

*TAQMAN Optimization.* The reactions for optimization were conducted as recommended for the TAQMAN Universal PCR Master Mix (Applied Biosystems) and as reported by Valsesia et al (37). The initial reaction conditions were the optimal SYBR green reaction. As before, a two-step PCR was tested. Primer concentrations were tested individually for the Af2, Zmt3 and INCW2-97 primers at 200, 75 and 50 nM, with probes at 200 nM. When the appropriate primer concentrations were identified, the probes were tested at 50, 100, 150, 200 and 250 nM. After probe and primer concentrations were optimized, multiplex reactions were tested using 75 nM Af2 primers, 200 nM Af2 probe, 75 nM Zmt3 or INCW2-97 primers and variable Zmt3 or INCW2-97 probe concentrations.

**qPCR Reproducibility Assays** . *SYBR Green Reproducibility.* DNA was extracted from four samples of ground maize for which aflatoxin concentrations had been previously determined (as described below). For each of the samples, six independent sub-samples of approximately 100 mg were extracted in a single 96 well plate.

Optimal conditions (see results section) for the SYBR green reaction were used with one of the extracted samples for each aflatoxin concentration. DNA was diluted

10 fold to have total DNA concentrations between 1 and 100 ng/µl. Each qPCR plate contained eight replicates of each sample and a duplicated standard (0.1 ng/µl pathogen DNA in 1 ng/µl host DNA). There were two wells for each sample, one with Af2 primers and another with Zmt3 primers. The qPCR experiment was conducted four times. Ct values for each plate were corrected based on the standard sample by adding a dCT, where $dCT=CT_{st}$ (value at which the standard curve is 0.1 for pathogen, or 1 for host) – $Ct_{pl}$ (average CT on each plate for the standard sample; 37). Using the standard curves obtained previously from the mixed DNA samples, the corrected CT value was transformed into DNA concentration. A ratio of pathogen to host DNA (p/h) was obtained by dividing the pathogen DNA by the host DNA concentrations.

For statistical analysis, the p/h ratios were log transformed and the following mixed model was run on JMP 7 (SAS Institute Inc, Cary, NC): $\log p/h = P_i + B_{k[i]} + C_j$, where $P_i$ = the random effect of each plate; $B_{k[i]}$= the random effect of replicate within plate and $C_j$ = the fixed effect of sample.

*TAQMAN reproducibility.* Approximately 100 mg (estimated by volume) of infected ground kernels with four concentrations of aflatoxin contamination (including zero) were placed in three independent plates. DNA extraction was performed using the CTAB method indicated below and diluted 1:10 in water prior to use. In the qPCR reaction, each plate contained a set of mixed standard curves in duplicate with concentrations of 10, 1, 0.1, 0.01 and 0.001 ng/µl of *A. flavus* DNA diluted in 1 ng/µl

of maize DNA. Three replicates of the qPCR procedure (qPCR plates) were conducted for each DNA extraction plate. Concentrations of pathogen DNA for each sample were estimated using the ABI Prism 7000 SDS Software Version 1.0 (Applied Biosystems).

Because a multiplex reaction to estimate the amount of host and pathogen DNA in a single tube was not possible with the primers and probes tested in this study, an infection coefficient was calculated by obtaining the ratio of DNA estimated by qPCR to the amount of DNA estimated by PICO green (pathogen/total DNA). These values were log transformed prior to analysis. Data analysis was carried out in JMP 7 (SAS Institute Inc, Cary, NC) using the following model: $\log IC = \gamma_i + \tau_{j[i]} + \Gamma_k + \gamma\Gamma_{ik}$, where $\gamma_i$ was the random effect of DNA plate (or extraction), $\tau_{j[i]}$ was the random effect of technical replicate or qPCR plate within DNA plate, $\Gamma_k$ the fixed effect of each maize line, and $\gamma\Gamma_{ik}$ was the random interaction of the DNA extraction by maize sample.

**Infection Coefficients in 20 Hybrids.** The experiment was set up in the field in a randomized complete block design with four blocks. Samples from each of the four biological replicates were placed in a 96-well plate three times for three independent DNA extractions. Each DNA plate was qPCR analyzed with three technical replicates. The qPCR settings and determination of the infection coefficient were as indicated for the optimal TAQMAN reaction. Infection coefficients were log transformed. Data analysis was carried out in JMP 7 (SAS Institute Inc, Cary, NC)

using the following mixed effects model: $\log IC = \beta_i + \gamma_j + \omega_{k[j]} + \beta\gamma_{ij} + \Gamma_l + \beta\Gamma_{il} + \gamma\Gamma_{il} + \Gamma\omega_{lk[j]} + \beta\omega_{ik[j]} + \beta\gamma\Gamma_{ijl}$, where $\beta_i$ was the random effect of biological replicate, $\gamma_i$ was the random effect of DNA extraction plate, $\omega_{k[j]}$ was the random effect of technical replicate (qPCR plate within DNA extraction plate); $\beta\gamma_{ij}$ was the random interaction of biological replicate by DNA extraction plate; $\Gamma_l$ was the fixed effect of maize hybrid; $\beta\Gamma_{il}$ and $\gamma\Gamma_{il}$ were the random interactions of hybrid by biological replicate and DNA extraction plate; $\Gamma\omega_{lk[j]}$ and $\beta\omega_{ik[j]}$ were the random interactions of maize hybrid and biological replicate by technical replicate within DNA extraction plate; and $\beta\gamma\Gamma_{ijl}$ was the random three-way interaction of biological replicate by DNA extraction and by maize hybrid.

Aflatoxin concentrations were obtained using the VICAM AflaTest (Watertown, MA) from 50 g of the same samples used for qPCR. For the ANOVA, aflatoxin concentrations were log transformed.

**Infection Coefficients in 18 Diverse Inbreds.** This experiment was established in the field in a randomized complete block design with 3 blocks. DNA was extracted from ground kernels from each line once. qPCR was conducted as indicated for the optimal TAQMAN reaction and replicated three times. Infection coefficients were log transformed. Data analysis was carried out in JMP 7 (SAS Institute Inc, Cary, NC) using the following mixed effects model: $\log IC = \beta_i + \omega_k + \Gamma_l$, where $\beta_i$ was the random effect of biological replicate, $\omega_k$ was the random effect of technical replicate and, $\Gamma_l$ was the fixed effect of maize inbred.

**Plant Materials and Fungal Inoculation**. For the development of the qPCR and tests of its reproducibility, four samples of ground maize kernels with 0, 60, 630 and 2320 ng/g aflatoxin contamination were used (Table 1). For all inoculations, *A. flavus* isolate NRRL 3357 was seeded onto 50 g of sterile maize cob grits with 100 ml of $H_2O$ and incubated at 28°C for 3 weeks. Before adjusting the concentration of the inoculum, the suspension was filtered through four layers of cheesecloth. The side-needle technique was used in which 3.4 ml of a suspension of $3x10^8$ conidia per ml was injected underneath the husk into the side of the top ear seven days after 50% of the silks had emerged on each row (44).

In order to study the correlation of aflatoxin concentration and *A. flavus* colonization, two sets of maize lines were inoculated. First, a set of 19 hybrids developed at the USDA-ARS Corn Host Plant Resistance Research Unit breeding program and a commercial hybrid Pioneer Brand 3394 were field inoculated as explained above at the Mississippi State field station. This experiment was planted in a randomized complete block design with four replicates. Second, 18 diverse inbreds (12, 21) were planted in a randomized complete block design with three replicates. Each line was planted in 4 m single-row plots spaced 0.97 m apart. Standard production practices for the region were followed (43). At harvest, the top ears of each plant in a row were dried at 38°C for seven days. Kernels from each row were ground with a Romer mill (Union, MO), and a subsample of 50 g was used for aflatoxin measurement using the VICAM AflaTest (Watertown, MA).

**DNA Extraction**. For the initial primer specificity tests and preparation of standard curves, *A. flavus* stock cultures (kept in 50% glycerol at -80°C) were streaked on potato dextrose agar plates (PDA, BD, Sparks, MD). Forty eight hours later, a single colony was selected and plated on a PDA plate. Three to five days later, abundant conidia were harvested by rinsing the plate with 5 ml of GYEP broth (20) and transferring the conidial suspension to a plate with 8 ml of GYEP broth. These plates were incubated for two days at room temperature. Mycelia were separated on filter paper (Whatman #1) with the help of a vacuum pump and rinsed twice with sterile water. Mycelia were then scraped with a sterile plastic loop into 2 ml microcentrifuge tubes. Tissue was lyophilized and stored at -80°C until processing.

DNA extraction protocols were adapted from standard methods (10). For the standard curve preparation, lyophilized *A. flavus* tissue or maize kernels were ground with a pestle in a mortar containing liquid nitrogen and placed in 2 ml tubes along with 1 ml of cetyltrimethylammonium bromide (CTAB) extraction buffer (2% CTAB, 1.4M NaCl, 100 nM Tris at pH 8, 20 nM EDTA at pH 8 and 0.1% 2-mercaptoethanol). Samples were agitated with a vortex mixer for 10 seconds and inverted twice before incubation at 60°C for 10 min. DNA was extracted twice with 700 µl of phenol:chloroform:isoamyl-alcohol 25:24:1, and centrifuged for 5 min at 12000 x g. The supernatant was transferred to clean tubes. DNA was precipitated with 100% ethanol and centrifuged for 5 min at 600 x g, and then again with 75% ethanol. Pellets were dried and resuspended in 100 µl of $H_2O$ (ELGA ultrapure water system, High

Wycombe, UK). DNA concentration was determined with a spectrophotometer (Biophotometer, Eppendorf, Hamburg, Germany).

Prior to DNA extraction, *A. flavus*-infected dry maize kernel samples (ground and prepared for aflatoxin determination) were kept at 4°C until processed. Approximately 100 mg of infected ground kernels were placed in 1.2 ml polypropylene Costar cluster tubes (Corning Inc., Corning, NY) that contained stainless steel 5/32" grinding balls (OPS Diagnostics, Lebanon, NJ) in 96-well racks. Plates were homogenized in GENO/GRINDER 2000 (SPEX CertiPrep Inc., Metuchen, NJ) at 550 strokes per min for 40 seconds. Plates were transferred to liquid nitrogen and then homogenized again. CTAB extraction buffer (500 µl per sample) was added and plates were incubated at 60°C for 5 min. Samples were mixed by inverting the plates 70 times, and then incubated at 60°C for 10 min. Cloroform : isoamylalcohol (24:1) was then added in two rounds for a total of 400 µl, and the plates were inverted 70 times. Plates were then centrifuged at 3000 x g for 12 min at 4°C. The supernatant was transferred to clean tubes and 300 µl of isopropanol (-20°C) were added. The plates were mixed by inverting 15 times and then chilled for a minimum of 1 hour at -20°C. Plates were then centrifuged at 1700 x G for 15 min at 4°C. Three hundred µl of 70%, and then 90% ethanol was added to the samples and centrifuged at 5890 x G. The pellets were dried and resuspended in 100 µl of nanopure $H_2O$. DNA was quantified using Picogreen (Invitrogen Corp., Carlsbad, CA) in a SPECTRAFLUOR PLUS fluorometer (Tecan US Inc., Research Triangle Park, NC) as indicated by the manufacturer's protocols.

***Results***

**Quantitative real-time PCR optimal conditions.** *SYBR Green Reaction.* Optimal primer concentrations were 200 and 75 nM for the Af2 forward and reverse primers respectively. Power SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA) was used at 1X concentration with 3 µl of sample template (~1-100 ng/µl) in 25 µl reactions. The most successful PCR conditions were 95°C for 10 min and 40 cycles of 95°C for 30s, 59°C for 30s and 72°C for 30s. For the SYBR green reactions, maize primers were used to estimate the amount of host DNA present in the sample in separate reactions under the same conditions but with Zmt3 primers at concentrations of 50 nM. There was a consistent dimer formation of the Zmt3 primers in the presence of *A. flavus* DNA.

**SYBR Green Standard Curves.** Af2 primers had a good linear relation with 111% efficiency within 10 ng/µl to 0.001 ng/µl range (Fig. 1A). The efficiency of these same primers was 98% in the pathogen-only DNA sample if considering only DNA concentrations from 10 to 0.01 ng/µl. In the standard curves from mixed DNA samples, amplification was detected only from 10 to 0.01 ng/µl and the efficiency of the reaction was 76%. This was the curve used for the SYBR reproducibility assays with regression function: y=31.34-4.04(x) and $R^2$=0.97. Using the Af2 primers, there was no detectable amplification of maize DNA, but a small dimer band was visible at the lowest maize DNA concentrations. Dissociation curves confirmed the occurrence of nonspecific amplification.

For maize primers Zmt3, there was a strong dimer band visible on agarose gels when amplification was conducted in the presence of *A. flavus* DNA (not shown). The reaction efficiency of the PCR from 100 to 0.01 ng/µl was 130% (Fig. 1B). This value is outside the accepted range (100% ±10). In spite of this, for the mixture of *A. flavus* and maize DNA, CT values for the mixed curve were almost exactly those of the host standard curve at 1 ng/µl, supporting the validity of the assay. At the maize DNA concentrations expected to be in samples for quantification (between 1 and 100 ng/µl), we consider the use of these primers to be acceptable. For the SYBR reproducibility assays, the maize standard curve was used only in the range of 100 to 1 ng/µl, which had an efficiency of 101% (the regression line was y=33.61-3.29 [x]).

**TAQMAN Reaction.** Optimal primer and probe concentrations were 75 nM for Af2 forward and reverse primers and 200 nM for the Af2 probe. As before, 3 µl of DNA template was used along with 1X PERFECTA qPCR SUPER MIX, UNG, ROX (Quanta Biosciences, Inc. Gaithersburg, MD) or TAQMAN Universal PCR Master Mix (Applied Biosystems, Foster City, CA). Twenty-five microliter reactions were carried out with a profile of 2 min at 50°C, 10 min at 95°C and 40 cycles of 95°C, 59°C and 72°C for 30 seconds each. The Zmt3 primers and probe produced nonspecific amplification when used in tandem and thus were not used for the TAQMAN reactions.

**TAQMAN Standard Curves.** The standard curves indicated that the Af2 primers and probe worked very well; a linear relation was found with an efficiency of 101.5% and $R^2$=0.99 (Fig. 1C). This reaction was repeated twice with four replicates each and similar results were obtained. When using mixed standard curves of *A. flavus* DNA diluted in 1 ng/µl of maize DNA, the efficiency of the Af2 detector was reduced to 61.4% ($R^2$=0.99) and the detection only occurred from 10 to 0.1 ng/µl of *A. flavus* DNA (Fig. 1D). Fungal biomass was estimated using the curve of *A. flavus* diluted in 1 ng/µl of maize DNA that had the following a regression line: y= 34.55 - 4.81(x).

The standard curves using the Zmt3 primers and probe for the mixed standard curves clearly showed nonspecific interaction of the Zmt3 detector with *A. flavus* DNA, and they were not used for further analysis. Other detectors were also tried, but the INCW2-97 primers and probe used for maize (26) did not work in tandem reactions with the Af2 primers and probe.

**Reproducibility Assay.** *SYBR Green Reproducibility.* There were no significant differences for the total DNA concentration of the samples with 2320, 630, 60 and 0 ng/g aflatoxin concentration ($P$= 0.517; mean 128.1 ng/µl, data not shown). In the qPCR experiments, technical replicates were a significant source of random variance while the replicates of the samples within a plate were not a significant source of variation. Significant differences ($P$<0.0001) for the p/h ratio were found among samples with different concentrations of aflatoxin contamination (Table 1, Fig. 2).

The correlation between p/h DNA ratio and aflatoxin concentration was 0.98 in 95 samples ($P$<0.0001).

*TAQMAN Reproducibility.* Multiple DNA extraction plates were included in this experiment. The analysis of variance showed that DNA extractions did not contribute significantly to the random variance of the experiment. However, technical replicate (qPCR plate), similarly to the results in the SYBR Green reaction, were a significant source of random variance (51 and 53% of the total variance estimates respectively). Significant differences ($P$ <0.0001) were found for the fixed effect of maize sample with different aflatoxin concentrations (Table 1, Fig. 2B). The correlation between IC and aflatoxin concentration was 0.81 in 36 samples ($P$<0.0001).

**Fungal infection and aflatoxin concentration in 20 maize hybrids**. The maize hybrids tested showed a wide range of responses to inoculation with *A. flavus*, with aflatoxin measurements ranging from 19 to 1188 ng/g. Significant differences ($P$ <0.0001) were found for fungal biomass estimated as infection coefficient for the fixed effect of hybrid (Table 2). Differences among hybrids were also significant ($P$<0.0001) for aflatoxin concentration levels (Table 3). DNA extraction was not a significant source of random variation. However, technical replicates within DNA extraction plate and the interaction of hybrid by biological replicate were significant sources of random variation (Table 2). Pearson correlation between the infection coefficient determined by qPCR and aflatoxin concentration was 0.85 ($P$<0.0001; n=20; Fig. 3A).

**Fungal infection and aflatoxin concentration in 18 diverse inbreds.** Significant differences were found among the diverse inbred maize lines ($P<0.0001$) that showed a wide range of aflatoxin concentrations (94 to 22734 ppb; Table 4). The infection coefficients were also significantly different ($P<0.0001$) but the range of values was smaller than for aflatoxin. Pearson correlation between the infection coefficient determined by qPCR and aflatoxin concentration was 0.81 ($P<0.0001$; n=18; Fig. 3B).

***Discussion***

We have demonstrated that qPCR can be used to reproducibly quantify *A. flavus* infection in maize kernels. Although the SYBR Green technique was found to be acceptable, we favor the TAQMAN method because of the higher levels of efficiency and sensitivity obtained during the validation experiments. For this method, DNA is extracted from 100 mg of infected tissue. Optimized PCR reactions (25 µl) contained 75 nM Af2 forward and reverse primers, 200 nM Af2 probe and 1X PerfeCTa qPCR Super Mix, UNG, ROX (Quanta Biosystems) or TAQMAN Universal PCR Master Mix (Applied Biosystems). Three microliters of DNA template was added to each reaction. DNA concentration of each sample was determined using Picogreen and all the samples were diluted equally in order to have a maximum concentration of 100 ng/µl DNA. We did not adjust the template DNA concentration for each sample before the qPCR reaction. Each qPCR plate contained a set of mixed standard curves in duplicate with concentrations of 10, 1, 0.1, 0.01 and 0.001 ng/µl of *A. flavus* DNA diluted in 1 ng/µl of maize DNA. The thermal profile was 2 min at 50°C, 10 min at

95°C and 40 cycles of 95°C, 59°C and 72°C for 30 seconds each. Three replicates of the qPCR procedure (qPCR plates) were conducted for each sample. Concentrations of pathogen DNA for each sample were estimated using the ABI Prism 7000 SDS Software Version 1.0 (Applied Biosystems). Infection coefficients (IC) were calculated by dividing the amount of pathogen DNA by the total DNA for each sample.

We have shown that the levels of fungal biomass were strongly correlated with aflatoxin accumulation in two sets of field-inoculated maize lines representing a broad range of genetic diversity. This finding indicates that fungal biomass estimated by qPCR could be used to infer the concentration of aflatoxin and that aflatoxin concentration should reflect levels of fungal biomass. It is important to acknowledge that this conclusion is based on a limited sample of maize germplasm, and is not necessarily applicable to all maize lines under all environmental conditions. Other studies have found that maize genotype could affect the accumulation of aflatoxin as discussed below. In the future, it would be informative to apply our qPCR technique to a larger maize population to clarify the effect of maize genotypes on the production of aflatoxins by *A. flavus*.

The cost of qPCR in a well-equipped molecular biology laboratory is lower than immunocapture aflatoxin determination (e.g. VICAM AflaTest) but more expensive than other plate-based ELISA methods. Our technique is especially valuable for pathology and breeding programs in which *A. flavus* infection levels are under study.

The method may also prove useful for detection and quantification in soil samples. The use of this technique with other *Aspergillus* spp. should be tested prior to use.

qPCR is now commonly used in plant pathology as a detection method, for example to identify *Phytophthora* species in forests (31). It has also been suggested as a method for the assessment of host resistance to *Plasmopara viticola* in grapevine (37). Quantitative PCR has also been used successfully to monitor the progression of aspergillosis, caused by *A. fumigatus*, in human serum and mouse (1, 7). A previous study reported on the quantification of *A. flavus* in food by quantitative real time PCR, using primers to the *nor-1* gene in the aflatoxin producing pathway (23). For our study, we designed the *A. flavus* primers within the internal transcribed spacer region 1. This region is located between the 18S and 5.8S rRNA genes and it is estimated that there are approximately 100 copies per genome (16). For this reason, we obtained high sensitivity demonstrated by the detection of 1 pg of *A. flavus* DNA in the standard curves. Cruz and Buttner (9) used primers designed in the ribosomal DNA genes and the ITS2 to successfully differentiate *A. flavus* grown in culture from 36 other fungal species grown on Petri plates as well as human and bacterial DNA. Unfortunately, by the time our study was completed we were not aware of their results so we did not use their primers in our study. In our article, we demonstrated the value of the method by identifying maize hybrids that allow significantly lower levels of *A. flavus* biomass estimated as an infection coefficient. The use of this method and primers provide a tool for the detailed study of the infection process and its relationship with aflatoxin accumulation. For example, the early rates of colonization

could be studied in different maize lines by dissecting infected tissue and analyzing by qPCR. Along this line, we are successfully using the TaqMan technique to map resistance QTLs affecting the early infection process in developing kernels and silk tissue.

Several pairs of *Z. mays* specific primers failed to produce reliable results and thus we were unable to create a multiplex TAQMAN reaction in which both host and pathogen DNA could be quantified from the same sample. The maize alpha tubulin genes designed for this study had problems of dimer formation but performed acceptably for the SYBR green reaction. However, for the TAQMAN reaction the PCR amplification was nonspecific, perhaps due to the addition of the probes. Murray et al. (26) reported on a qPCR technique used to estimate maize endogenous DNA degradation using maize specific primers and TAQMAN probes in the cell wall invertase gene (INCW primers). These primers and probe were quite specific when using pure maize DNA from the inbred line B73, but did not work when used in tandem (host plus pathogen) PCR reactions. Results not shown from the optimization assays indicated that the addition of maize DNA at higher concentrations (100 instead of 1ng/µl) to the *A. flavus* standard curve had the effect of delaying the reaction instead of affecting the efficiency when using the Af2 primers and probe. This indicates that variable amounts of maize DNA do not affect the accuracy of the quantification. Adding the INCW primers, in contrast, had a negative effect on the efficiency of the Af2 detector, and this would make for an inaccurate quantification. Possible causes for this are the amplification of non-target regions or primer

interactions with other nucleic acids that compete with the amplification of the specific product. In addition, *A. flavus* isolates have been reported to have PCR inhibitors (9). In the end, it was concluded that the most accurate quantification of fungal biomass in maize lines with diverse genetic backgrounds would be achieved by using only the *A. flavus* specific primers and probe with a standard curve of pathogen DNA diluted in 1 ng/µl of maize DNA in each plate.

Through a series of reproducibility assays and an experiment involving multiple biological and technical replicates, we were able to determine the most important effects for biomass determination using qPCR. We found that DNA extraction and its interaction with hybrid lines, as well as replicates within a plate, did not add significant levels of variance to our experiments. These results suggest that, with our methods, DNA extraction or the number of replicates within a plate does not affect the outcome of the biomass estimation. On the other hand, technical replicates are a source of variation, so multiple qPCR plates must be run in order to obtain accurate results. Significant sources of random variance were found for all the interactions involving biological replicates. Aflatoxin concentrations in field experiments are highly variable, and our results suggest that this variation is due to how extensively maize kernels are colonized as opposed to how much toxin is produced for a given degree of colonization.

A strong correlation between *A. flavus* biomass and aflatoxin accumulation was found among the maize genotypes tested in this experiment. In another study,

conducted using a GFP-producing isolate, a similar correlation (0.85) was found between fluorescence and aflatoxin concentration in cotton seed (29). A good correspondence in ranks between visual GUS ratings and aflatoxin concentration was also reported from five maize lines inoculated with a GUS transformed isolate (4). These results are not consistent with the idea that pathogen load and aflatoxin accumulation are distinct traits in maize, at least with the germplasm and conditions used here. However, it is important to point out that there is evidence of conditions that allow fungal growth with low aflatoxin contamination. Using a GUS expressing *A. flavus* strain, Brown et al (5) showed that one African aflatoxin resistant inbred maize line (1368) allowed high levels of infection while two inbred maize lines (1188 and 15) with high aflatoxin contents supported low levels of infection. The set of inbreds tested was selected to represent a relatively broad genetic diversity. Our panel of hybrids represented a limited sample of maize diversity but a wide range of quantitative responses in accumulation of aflatoxin concentration and pathogen biomass. The parents of the hybrids include three inbreds released as sources of resistance to aflatoxin accumulation: Mp313E, Mp715, and Mp92:673 (released as Mp717) (32, 41, 42). In addition, the line Mp494 has also been shown to have significant general combining ability for resistance to aflatoxin accumulation (43). Not surprisingly, crosses involving these lines had significantly lower levels of *A. flavus* biomass and aflatoxin contamination. All of these resistant lines have the open pollinated cultivar Tuxpeño (reported as Tuxpan) in their pedigrees and thus it is possible that we are assessing a single source of resistance. The use of a common source of resistance may account for the high correlation between infection levels and

aflatoxin contents. While the sources of resistance tested in the African maize germplasm may be different, it is possible that prior conclusions have been affected by the use of methods that do not permit accurate measurement of pathogen biomass. Interestingly, among our inbreds, the line IBM262 allowed high levels of aflatoxin contamination while the infection coefficient levels were indistinguishable from the most resistant lines. Currently our inbreds are being tested again in the field to confirm our results. It is also possible that the environment has differential effects on fungal growth and aflatoxin biosynthesis. More studies are needed to further explore the effects of defined host genes on colonization and contamination of maize by *A. flavus*.

**ACKNOWLEDGMENTS**

Funded in part by The McKnight Foundation and the Generation Challenge Program. Thanks to E. Helliwell for advice on qPCR development and to the Cornell Statistical Consulting Unit for advice on data analysis. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by USDA.

**Table 1.** Reproducibility assays for the SYBR green and TaqMan reaction assays for *A. flavus* biomass estimated as pathogen to host DNA ratio (p/h) or infection coefficients (IC) on aflatoxin contaminated kernel samples.

| Sample | Aflatoxin (ng/g) | SYBR green assay | | | Taqman assay | | |
|---|---|---|---|---|---|---|---|
| | | log p/h | | p/h % | log IC | | IC |
| High | 2320 | -1.35 | a[z] | 26.0 | 4.70 | a[z] | 109.4 |
| Medium | 630 | -2.70 | b | 6.7 | 2.50 | b | 11.1 |
| Low | 60 | -5.40 | c | 0.5 | 1.21 | c | 2.4 |
| Control | 0 | -8.72 | d | 0.0 | 0.03 | d | 0 |

[z] Levels not connected by same letter are significantly different as determined by Tukey test (α=0.05).

**Table 2.** F tests for the fixed and random effects[a] on the infection levels estimated by qPCR of 20 hybrids field inoculated with *A. flavus*.

| Source | df | F statistic | *P* |
|---|---|---|---|
| Biological replicate (BR) | 3 | 0.8919 | 0.4808 |
| DNA extraction (DE) | 2 | 1.5053 | 0.2777 |
| Technical replicate (TR) within DE | 6 | 5.9038 | 0.0013 |
| BR x DE | 6 | 1.9561 | 0.0919 |
| Hybrid | 19 | 7.7169 | <.0001 |
| Hybrid x BR | 57 | 2.0504 | 0.0006 |
| Hybrid x DE | 38 | 0.6776 | 0.9151 |
| Hybrid x TR[DE] | 114 | 1.1987 | 0.1100 |
| BR x TR[DE] | 18 | 7.0273 | <.0001 |
| BR x DE x hybrid | 114 | 5.7637 | <.0001 |

[a] See text for details on the model.

**Table 3.** Differences between *A. flavus* biomass infection coefficient (IC) determined by qPCR and aflatoxin concentration for maize hybrids inoculated in the field.

| | *A. flavus* | | | Aflatoxin | | |
|---|---|---|---|---|---|---|
| **Pedigree/Line** | **log (IC+1)** | | **IC** | **log (ng/g +1)** | | **ng/g** |
| P3394 | 3.67 | a[z] | 38.1 | 7.08 | A | 1188 |
| (CH05015:n12-43-1-B-B)-3-2 x T173 | 3.47 | ab | 31.2 | 6.75 | ab | 850 |
| Mp97:154 x T173 | 2.98 | abc | 18.7 | 6.70 | ab | 812 |
| GA209 x SC212M | 2.71 | abcd | 14.0 | 6.83 | ab | 922 |
| (Mp313E x Va35 Fam 58)-2-3-1-1-2-2 x T173 | 2.45 | abcde | 10.6 | 5.46 | abcde | 235 |
| CML322 x T173 | 2.31 | abcde | 9.1 | 6.56 | abc | 705 |
| CML326 x T173 | 2.31 | abcde | 9.0 | 6.39 | abcd | 597 |
| Mp420 x T173 | 2.22 | abcde | 8.2 | 6.02 | abcde | 412 |
| CML342 x T173 | 2.15 | abcde | 7.6 | 6.26 | abcd | 522 |
| (MBR-ET WHITE F2-112-1-1xB-B-#-B-#)-1-2 x T173 | 1.96 | bcde | 6.1 | 6.53 | abc | 687 |
| Mp97:161 x T173 | 1.56 | cde | 3.7 | 5.59 | abcde | 266 |
| CML247 x T173 | 1.43 | cde | 3.2 | 4.39 | cdef | 79 |
| Mp92:673 x T173 | 1.34 | cde | 2.8 | 4.30 | def | 73 |
| (Mp 715 x Va35)-1-3-4-2-1 x T173 | 1.18 | de | 2.3 | 5.10 | abcdef | 163 |
| CML348 x T173 | 1.18 | de | 2.3 | 5.05 | abcdef | 156 |
| Mo18W x Mp313E | 1.09 | de | 2.0 | 5.06 | abcdef | 156 |
| Mp494 x Mp92:673 | 1.03 | de | 1.8 | 3.22 | F | 24 |
| (MBR-ET WHITE F2-112-1-1xB-B-#-B-#)-1-1 x T173 | 1.03 | de | 1.8 | 4.77 | bcdef | 117 |
| Mp313E x Mp715 | 0.92 | e | 1.5 | 2.99 | F | 19 |
| Mp494x Mp715 | 0.85 | e | 1.3 | 3.89 | ef | 48 |

[z] Levels not connected by same letter are significantly different as determined by Tukey test (α=0.05).

**Table 4.** Differences between *A. flavus* infection coefficient (IC) determined by qPCR and aflatoxin concentration for a panel of diverse maize inbreds inoculated in the field.

| | *A. flavus* | | | Aflatoxin | | |
|---|---|---|---|---|---|---|
| **Pedigree/Line** | **log (IC+1)** | | **IC** | **log (ng/g+1)** | | **ng/g** |
| CML103 | 0.78 | a | 1.18 | 10.03 | a | 22735 |
| Mo17 | 0.62 | ab | 0.85 | 9.39 | ab | 11982 |
| B73 | 0.51 | abc | 0.67 | 9.54 | ab | 13917 |
| B97 | 0.33 | bcd | 0.39 | 9.22 | abc | 10118 |
| MS71 | 0.28 | bcd | 0.33 | 8.21 | abcde | 3689 |
| Oh43 | 0.27 | bcd | 0.31 | 7.84 | bcde | 2542 |
| Oh7B | 0.21 | cd | 0.23 | 8.16 | abcde | 3483 |
| IBM54 | 0.11 | d | 0.12 | 7.93 | bcde | 2790 |
| NC358 | 0.11 | d | 0.11 | 6.75 | defg | 854 |
| IBM262 | 0.09 | d | 0.10 | 8.75 | abcd | 6283 |
| Ky21 | 0.08 | d | 0.08 | 6.96 | defg | 1056 |
| Tx303 | 0.06 | d | 0.06 | 7.20 | cdef | 1333 |
| Ki3 | 0.05 | d | 0.06 | 7.46 | bcde | 1739 |
| Mp339 | 0.05 | d | 0.05 | 6.79 | defg | 886 |
| M37W | 0.02 | d | 0.02 | 6.43 | efgh | 622 |
| CML52 | 0.00 | d | 0.00 | 4.55 | h | 94 |
| Mp313E | 0.00 | d | 0.00 | 4.84 | gh | 126 |
| CML247 | 0.00 | d | 0.00 | 5.15 | fgh | 172 |

[z] Levels not connected by same letter are significantly different as determined by Tukey test ($\alpha$=0.05).

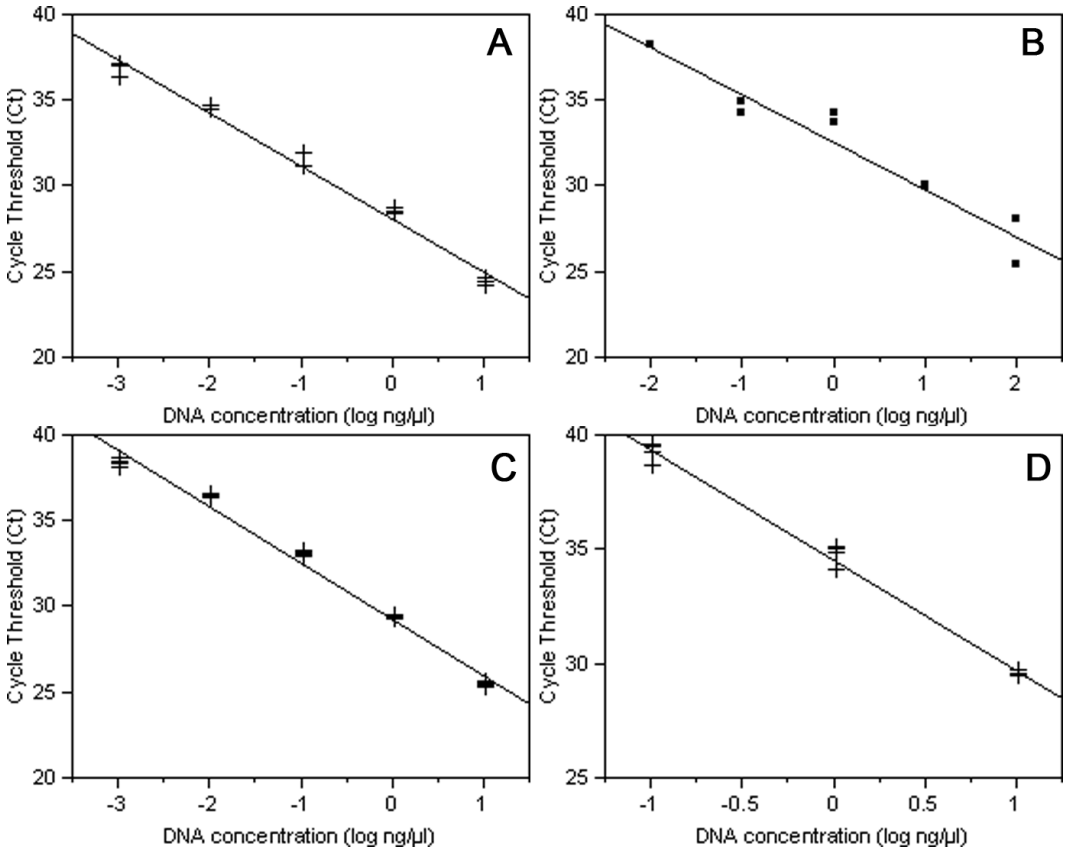


**Figure 1.** Standard Curves A) SYBR green reaction with *A. flavus* DNA and Af2 primers, efficiency: 110; $R^2$=0.98; y = 28.12 - 3.08(x). (N=3). B) SYBR green reaction with maize DNA and Zmt3 primers, efficiency: 130; $R^2$=0.93; y = 32.57 - 2.75(x). (N=2). C) TaqMan reaction with *A. flavus* DNA and Af2 primers and probe, efficiency 101; $R^2$=0.99; y= 29.25 - 3.29(x) (N=4). D) TaqMan reaction with mixed DNA and Af2 primers and probe efficiency: 61.5; $R^2$=0.99; y = 34.55 – 4.81(x). (N=3).

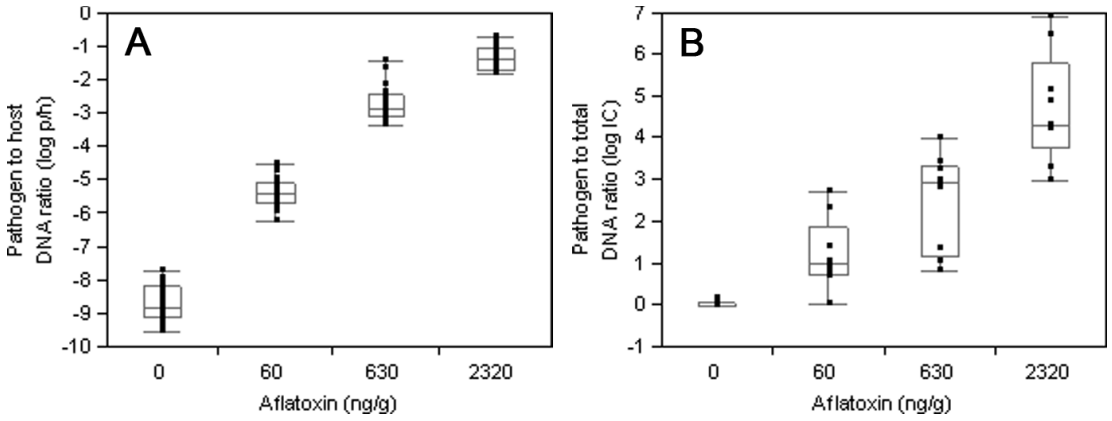


**Figure 2**. Reproducibility assays. A) SYBR green reaction for three samples of ground maize kernels infected with known concentrations of aflatoxin contamination and a control. A ratio of the pathogen to host DNA (p/h) was obtained. Significant differences were found among treatments ($P$<0.0001). The correlation between p/h and aflatoxin concentration was 0.98 ($P$<0.0001, N=95). B) Taqman reaction for the same four samples. An infection coefficient (IC) was calculated by dividing the amount of DNA estimated with qPCR by the amount estimated with PicoGreen (pathogen/total DNA). Significant differences were found for the category effect ($P$ <0.0001). The correlation between IC and aflatoxin ppb was 0.81 (P<0.0001, N=36).

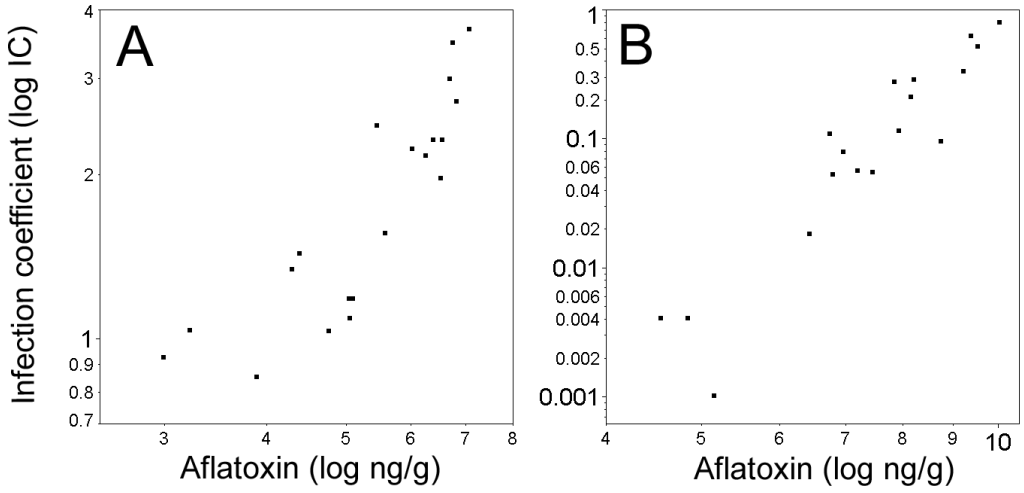


**Figure 3.** A) Correlation (0.85; P<0.0001) between fungal biomass estimated as infection coefficient (IC) and aflatoxin concentration for 20 field inoculated maize hybrids. The field experiment had four biological replicates. IC was estimated by TaqMan qPCR from three independent DNA extractions, and each DNA sample was analyzed three times (technical replicates). IC was calculated by dividing the amount of *A. flavus* DNA obtained from qPCR by the total DNA present in the sample. B) Correlation (0.81; *P*<0.0001) between infection coefficient (IC) and aflatoxin average levels for a set of diverse maize inbreds (N=18). The field experiment had three replicates, and the qPCR experiment was conducted three times (technical replicates). IC was calculated as indicated for the TaqMan reproducibility assay.

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# CHAPTER 3

# TISSUE-SPECIFIC COMPONENTS OF RESISTANCE TO ASPERGILLUS EAR ROT OF MAIZE

## *Introduction*

Aflatoxins are fungal secondary metabolites produced by several species from the genus *Aspergillus*. In maize the most common causal agent of aflatoxin contamination is *Aspergillus flavus*. Aflatoxin accumulation in maize occurs in the field (pre-harvest) and in storage (post-harvest). Control measures could range from regulatory enforcement to detoxification in the diet. In this study we focus on the characterization of maize resistance to aflatoxin accumulation. There are several reports of maize inbred lines that accumulate lower levels of aflatoxin, this is resistant lines (3, 12, 24, 26). The mechanisms by which maize plants accumulate lower levels of aflatoxin are unknown. A confounding factor for the use of this resistance is that year-to-year accumulation of aflatoxins is highly variable and weather conditions, particularly temperature and humidity, strongly affect aflatoxin contents (27).

The genetic basis of resistance has been studied using diallel studies and QTL mapping populations. Early studies indicated the absence of complete resistance and point to a strong importance for general combining ability, which suggests that the genetic effects are additive. However there are some reports where specific combining ability is the main effect, pointing to the presence of dominant or epistatic gene effects (3, 6, 12, 25). More recent QTL mapping experiments confirm the

importance of additive genetic effects and indicate the existence of at least 14 regions of the genome where QTL from multiple studies co-localize. Most of these QTL have small additive effects (Chapter 5).

In an influential review, Parlevliet (18) divided rate-reducing resistance, also known as incomplete or quantitative resistance, into resistance to infection, colonization, and reproduction. Since then there have been many technical advances; for example, Chapter 2 describes the development of quantitative real-time PCR to accurately measure the levels of *A. flavus* colonization (17). Further advances come from QTL mapping studies that dissect quantitative resistance into multiple causal loci. A few QTL for quantitative resistance in plants have been cloned (23) and many genes have been implicated in resistance through genome-wide association studies (14, 20), suggesting that diverse host functions contribute to quantitative disease resistance. A detailed microscopic analysis of components of quantitative resistance using near-isogenic maize lines containing different QTL for resistance to *Setosphaeria turcica* recently proved that, while one QTL reduces infection efficiency, the other limits colonization (7). Therefore, we expect that the multiple genes that underlie quantitative resistance to *A. flavus* affect different components of resistance.

No previous studies of the components of resistance to *A. flavus* in maize have been published. Aflatoxins are not known to play a part in the pathogenesis of *A. flavus* on maize, yet aflatoxin accumulation is the main target for studies of maize resistance to *A. flavus* because of the importance of this trait in food systems.

However, analysis of aflatoxin levels in multiple lines is time consuming and costly. Thus other traits associated with the disease have been used as proxies for aflatoxin accumulation, for example: bright greenish yellow fluorescence (BGYF) and ear rot severity. The correlation of BGYF with aflatoxin accumulation has ranged from 0.59 to 0.79 in different studies, but since this trait seems to vary depending on the source of resistance it was not recommended for selection of resistant lines (6). Ear rot severity has also been studied but its correlation with aflatoxin accumulation varied from a significant 0.53 in one year to no correlation in the second year for one population (6) or was significantly stable between 0.41 to 0.64 in two years for another population (15).

Tissue specific resistance may also play a role in resistance to *A. flavus*, because in the absence of insect damage, the pathogen colonizes the silk before proceeding into the maize kernels (22). For *Ustilago maydis*, another maize pathogen, different QTL were found associated with resistance in various maize tissues (2). Even though silk resistance to infection by another ear rot pathogen (*Fusarium graminearum*) has been clearly characterized (21), evidence for silk resistance to *A. flavus* is indirect (19, 27). Finally, several authors have pointed out that aflatoxin accumulation may be related to other kernel traits such as grain composition or plant traits such as husk or pericarp (12). Two studies have found significant correlations between aflatoxin accumulation and traits such as grain texture, husk cover, grain yield and silk channel length (1, 3).

In order to dissect the highly variable phenotype by looking for resistance at the multiple steps of the plant-fungal interaction, we used a panel of diverse inbred maize lines and evaluated four components of silk resistance and six components of kernel resistance in replicated experiments over three years. We conducted *in-vitro* and field inoculations with the objective of directly challenging the two plant tissues and to better test the various components of resistance. We hypothesized that natural variability exists in maize for colonization in silk and kernel tissues. It was expected that some of these traits would be less variable than aflatoxin accumulation. Finally, in order to know if any of the components is a reliable predictor of aflatoxin accumulation, we conducted a correlation analysis and included other traits available for the panel of diverse maize lines.

***Materials and Methods***

**Plant and Fungal Materials.** For a preliminary experiment, seven inbreds were planted in single pots in a greenhouse during the winter of 2007 in Ithaca NY (Table 6). Up to twenty six maize inbreds (Table 2) selected by others to maximize their diversity (11) or because they are sources of resistance or susceptibility to aflatoxin accumulation were planted in 2007, 2008 and 2009 at Cornell's Robert Musgrave Research Farm in Aurora, NY and in 2008, 2009 and 2010 at the R. R. Foil Plant Science Research Center at Mississippi State University, MS (MSU).

Inoculum for *in-vitro* procedures was prepared by growing *A. flavus* isolate NRRL 3357 on 20 g of corn kernels. Prior to inoculation, the corn kernels were soaked overnight with 10 ml of $H_2O$ in 500 ml flasks and then autoclaved. Conidia

were washed 12-18 days after inoculation with 20 ml of distilled $H_2O$ containing 0.2% Tween 20. Conidia concentration was adjusted to $1x10^7$ conidia per ml, with a hemocytometer.

For field inoculations, *A. flavus* isolate NRRL 3357 was seeded onto 50 g of sterile maize cob grits with 100 ml of $H_2O$ and incubated at 28°C for three weeks. Before adjusting the concentration of the inoculum to $3x10^8$ conidia per ml, the suspension was filtered through four layers of cheesecloth. Ears were inoculated seven days after 50% of the silks had emerged.

**Components of Silk Resistance.** To produce the test tissues for *in-vitro* inoculation, 12 kernels of each maize line were planted in single rows at Cornell's Robert Musgrave Research Farm in Aurora, NY. Five individual pots were planted for the greenhouse grow-outs. At anthesis, silks of four plants in a row were cut at the tip and the ears were covered with shoot bags. The next day, the newly emerged silks were sib or self-pollinated. One day after pollination, the tips of the ears, including the recently pollinated silks, were cut and transported to the laboratory on ice.

For each experimental unit (one plant), five silks in 2007 and 10 in 2008 and 2009 were placed in a Petri plate without a lid. Dishes were contained in culture trays lined with chromatography paper moistened with 30 ml of sterile $H_2O$ to maintain humidity. Four replicates of each line were prepared from the four plants in a row. Silks were inoculated with 10 µl in 2007 and 50 µl in 2008 and 2009 of *A. flavus* conidia prepared as indicated above. After inoculation, culture trays were placed in an incubator at 30°C for seven days in the dark.

For latent period (LP) rating, trays were observed daily under a dissecting microscope until the appearance of the first conidiophore-bearing yellow-green conidia. Sporulation rating (SP) was conducted seven days after inoculation with a dissecting microscope using a scale from 0 to 5 where 0 meant no spores and 5 was the highest density of conidiophores. Along with SP, infection frequency (IF) was rated as a percentage of the silk harboring conidiophores. When SP and IF rating were complete, the top three centimeters of the silks were cut and kept at -80°C until DNA extraction for colonization rating using qPCR as explained below.

Field inoculation was conducted in an environment conducive to aflatoxin accumulation at MSU. Inbred lines were planted in a randomized complete block design with three replicates. Each line was planted in four-meter single-row plots spaced 0.97 m apart. In order to measure components of resistance in the silk and in the kernels, both sites were inoculated by injecting 1.7 ml of the conidial suspension in the silk channel and 1.7 ml underneath the husk into the side of the top ear at seven days after mid-silk stage.

In order to determine the levels of silk colonization, two ears from each row were collected seven days after inoculation and transported to the laboratory on ice. Approximately 100 mg silk tissue from the ear channel was collected in 1.2 ml polypropylene Costar cluster tubes (Corning Inc., Corning, NY) and frozen until processing. Silk samples were lyophilized prior to DNA extraction. Colonization levels were determined using Taqman chemistry qPCR as described previously (Chapter 2)(17). Briefly, total DNA concentration was determined using Picogreen on all the samples. *A. flavus* DNA concentration was determined by comparing to a standard curve included in each PCR plate. Three replicates of the qPCR procedure

were conducted for each sample. The colonization was calculated by dividing the amount of pathogen DNA by the total DNA for each sample.

**Components of Kernel Resistance.** For *in-vitro* inoculation of developing kernels, each inbred planted in the field in Aurora NY was self or sib pollinated. Three weeks after pollination, a procedure similar to the kernel screening assay was conducted (4). Four ears were harvested from each inbred and five developing kernels per ear were placed in small Petri plates in culture trays lined with chromatography paper that had been moistened with 30 ml of sterile $H_2O$. Kernels were dip-inoculated in a conidial suspension of *A. flavus* isolate NRRL 3357 at $1x10^7$ conidia per ml, prepared as explained for the silk experiments. The four ears were considered technical replicates for each experiment.

Latent period and infection frequency were visually rated seven days after inoculation. All inbred lines and all kernels had at least some clearly visible spores three days after inoculation. Sporulation (SP) was rated on each kernel seven days after inoculation using a dissecting microscope as a percentage of the kernel covered with conidiophores. After SP rating, kernels were kept in envelopes at -80°C until processed by qPCR analysis for colonization estimation. Kernel samples were lyophilized prior to DNA extraction. Infection levels were determined using Taqman chemistry qPCR as described previously (17).

Field inoculation for kernel components of resistance was conducted as indicated for the silk experiments at MSU. For aflatoxin determination at harvest, the top ears of each plant in a row were dried at 38°C for seven days. Kernels from each row were ground with a Romer mill (Union, MO), and a subsample of 50 g was used

for aflatoxin measurement using the VICAM AflaTest (Watertown, MA). This same subsample of dried kernels was kept at 4°C until processed to determine the levels of colonization using Taqman chemistry qPCR.

**Statistical Analysis.** All statistical analyses were conducted on JMP V 8.0 (SAS Institute, Cary, NC). In all cases, the data was analyzed for the overall studies including year in a mixed model as a random variable, and on a year-by-year basis. For the combined analysis, only lines for which there was data available for at least two years were used, and the *in-vitro* inoculation with tissue from the greenhouse study was not included. In order to standardize the variances, silk LP and SP data were log transformed, while IF and colonization were arcsine square root transformed. Some of the developing kernel assays for LP and IF did not require a statistical analysis because all the kernels had visible sporangia at the same time. The levels of sporulation, however, presented significant variation. SP data on developing kernels was arcsine square root transformed prior to analysis. Finally, data from field inoculations in the kernel for colonization and aflatoxin accumulation was log transformed.

Pairwise correlations among components of resistance were conducted using the least squares means of each component of resistance for the three years of experiments and on a year-by-year basis. For comparison purposes, aflatoxin accumulation in kernels from field studies was included with the silk data correlations. Two other sets of data gathered on a similar panel of inbred lines were included for comparison: kernel traits reported by Flint-Garcia et al. (10), and days to silk (flowering time) reported by Buckler et al. (5).

***Results***

Significant differences in *A. flavus* colonization on silks and mature kernels as well as aflatoxin accumulation in mature kernels were detected among entries for three years of experiments (Tables 1 and 2). All the components of resistance were highly influenced by the environment with significant effects for the random variation due to the interaction between year and inbred line.

For silk tissue, *in-vitro* infection frequency and sporulation means were the only significantly correlated components of resistance. None of the components of silk resistance correlated with aflatoxin accumulation or with days to silk (Table 3). For kernel tissue, *in-vitro* inoculation of developing kernels was consistent within a year but was highly influenced by the environment. The interaction between year and inbred was the dominant source of variation, rendering differences between lines insignificant in the overall analysis (Table 1). However, at maturity in field inoculation experiments, we found significant differences in the panel of maize lines for colonization and aflatoxin accumulation (Tables 1 and 2). Aflatoxin levels and colonization were significantly correlated (0.70***) (Table 4). Aflatoxin accumulation was negatively correlated with, fiber and ash in kernels, while a positive correlation was found between aflatoxin with carbohydrate and seed weight. *In-vitro* sporulation levels on developing kernels were negatively correlated with moisture levels of mature kernels. There were significant correlations between days to silk (flowering time) and aflatoxin accumulation in the field as well as *in-vitro* sporulation of developing kernels (Table 4).

On a year-by-year basis, there were significant differences among inbred lines for 10 of the 14 components of silk resistance studied with *in-vitro* inoculation (Table

5). For two of them (latent period for field-grown material in 2007 and sporulation from greenhouse materials in 2007), despite the significant differences found in the analysis of variance, the differences among inbred lines were not discernible by Tukey test ($\alpha$=0.05). For field inoculation assays, we found significant differences for colonization among inbred lines for each of the three years of study. The ranking of lines varied from year to year for those components of resistance that were both significantly different with an analysis of variance and a multiple-test-controlling statistic (Tukey)(Table 6 and 7). None of the components of silk resistance was significantly correlated with aflatoxin accumulation on kernels (Table 8). Rankings for silk colonization from field inoculations in 2008, 2009 and 2010 were significantly correlated as determined by a significant Spearman correlation. In addition, the components of resistance in 2009 evaluated *in-vitro* were significantly correlated with each other, as were sporulation in 2009 and infection frequency in 2007. Rankings of latent period evaluated *in-vitro* from greenhouse materials were negatively correlated with infection frequency and sporulation in 2009, but positively correlated with colonization in 2008 (Table 8).

There were significant effects for inbred lines in every experiment for sporulation and colonization of developing kernels. *In-vitro* inoculations on developing kernels in four experiments indicated that there were no differences for latent period or infection frequency among the inbreds tested in this study. Conversely, in field inoculation experiments every year we found significant differences for colonization and aflatoxin accumulation (Table 5). Rankings for the components of kernel resistance also varied from year to year (Tables 9 and 10). Aflatoxin accumulation and colonization from field inoculations were generally correlated, with some exceptions with the 2010 data. Aflatoxin accumulation in 2008

was significantly correlated with sporulation from *in-vitro* inoculations in 2008 (0.59*) and by ranks in 2007 (0.37*). The ranks of *in-vitro* sporulation in 2008 were also correlated with colonization in the field in 2008. However, *in-vitro* sporulation rankings were negatively correlated with colonization in 2008 (-0.45*). Colonization for *in-vitro* inoculations with greenhouse materials was correlated with sporulation from the same materials (Table 11).

***Discussion***

Significant year-by-inbred interactions for every component of resistance studied indicated that the environment in which the plant is grown has a large effect on the expression of resistance. No component of resistance was stable across environments. *In-vitro* experiments kept the plants in a homogeneous environment and we still found significant variation from year to year, indicating that the environment in which the plant is grown determines the *A. flavus* - maize interaction. In addition, a significant negative correlation of flowering time with aflatoxin accumulation indicates that lines that are inoculated later in the season accumulate lower levels of aflatoxin. This could also be due to environmental variation towards the end of the season.

Despite the environmental variation, there is clear evidence in field inoculations of resistance to silk colonization by *A. flavus* in maize. Controlling for the interaction by including it in the mixed model, we still found significant differences among lines across three years for silk and kernel colonization and for aflatoxin accumulation in field inoculation experiments. A strong correlation (0.70***) between aflatoxin accumulation and colonization in field experiments confirms our previous results, in which the two traits were significantly correlated

(0.85) in the first year of this study (17). Although aflatoxin and silk colonization in the field were not correlated, it is important to keep in mind that our aflatoxin determination was done on kernels that were inoculated in the ear, by-passing any possibility of silk resistance. We have demonstrated the existence of silk resistance but the question remains about the importance of silk in natural aflatoxin accumulation.

Strong variation among years for *in-vitro* traits sharply contrasted with the reproducibility within any given year (Table 5). This again suggests that the environment in which the plants are grown affects the quality or composition of the kernel and silk tissue. With regard to *in-vitro* studies, our data also clearly indicate that there is no correlation with aflatoxin accumulation in the field, establishing the lack of utility of this type of trait for breeding efforts, and making this an important point when designing studies of the *A. flavus*, aflatoxin and maize interaction because the *in-vitro* results do not seem to be extendable to field applications.

The discrepancy among years for *in-vitro* experiments prompted us to explore kernel composition as a possible determinant for *A. flavus* colonization and aflatoxin accumulation. Flint-Garcia et al. (10) had determined kernel composition and seed characteristics in a panel that contained 19 of the inbreds used in this study. Significant correlation of aflatoxin content with fiber, ash, carbohydrates, and seed weight suggests that kernel composition traits should be further explored in the future. Surprisingly, kernel colonization was not significantly correlated with any kernel composition trait, although some correlation coefficients were relatively high (0.5). This finding suggests that while aflatoxin production may be influenced by kernel composition, infection of the kernel occurs regardless of the substrate. This

hypothesis is in agreement with the finding that some antioxidant compounds reduce aflatoxin production (13).

Another interesting significant negative correlation was found between kernel sporulation from *in-vitro* assays and kernel moisture. This is puzzling since the *in-vitro* assays were conducted using developing kernels and moisture was determined on ground kernels after harvest and drying (10). This suggests a testable hypothesis that structural features that prevent drying also prevent pathogen ingress. Supporting this hypothesis is the recent finding that *F. verticillioides* enters the kernel though the stylar canal and that stylar canal apertures vary between resistant and susceptible maize lines (9). It would be prudent to characterize the diverse panel of lines used in this study for the stylar canal size. Such a study would require scanning electron microscopy. It is also interesting to note that the microscopic analysis conducted on the initial infection of *A. flavus* and maize kernels were conducted a few decades ago and no recent detailed infection study such as the one conducted for *F. verticillioides* has been conducted.

In this chapter, compelling evidence is presented that, in addition to resistance to aflatoxin accumulation, there is variation in maize germplasm for susceptibility to silk and kernel colonization. We found that the environment in which the plants were grown heavily influenced *in-vitro* inoculation assays for *A. flavus*. We did not identify a component of resistance that is not environmentally affected but we found that kernel characteristics and flowering time were significantly associated with resistance to aflatoxin accumulation. Many of the lines used in this study are part of maize diversity sets or other large public efforts to characterize quantitative traits in maize

and our results could provide a guide for future hypothesis testing using these resources (11, 16).

**Table 1.** *P*-value for the effect of inbred line on multiple year evaluations of components of resistance to *A. flavus* in a diverse set of maize inbred lines[a].

| Component of resistance | | Maize Tissue | | |
|---|---|---|---|---|
| | | Silk | Developing kernel[b] | Mature kernel[b] |
| *In-vitro* | Latent Period | 0.3799 | ns[c] | |
| | Infection Frequency | 0.2270 | ns | |
| | Sporulation | 0.2390 | 0.0963 | |
| | Colonization (qPCR) | - | 0.2097 | |
| Field | Colonization (qPCR) | 0.0312 | - | 0.0301 |
| | Aflatoxin accumulation | - | - | 0.0006 |

[a] *In-vitro* experiments were replicated four times using field materials grown in NY for three years. Field inoculation experiments were replicated three times in a randomized complete blocks design for three years. For all the experiments except for silk latent period evaluation, there was a significant effect of the random variation due to the interaction of year by inbred line.
[b] *In-vitro* experiments on kernels were conducted on three week old kernels. Field evaluation of components of resistance was conducted on mature kernels.
[c] ns = not significant, no statistical analysis required.

**Table 2.** Least squares means for silk and kernel components of resistance in a panel of diverse maize inbreds inoculated for three years in the field.

| | Silk | | Mature kernel | | | |
|---|---|---|---|---|---|---|
| | Colonization (IC) | | Colonization (IC) | | Aflatoxin (ng/g) | |
| Mo17 | 0.14 | c[b] | 1.63 | ab | 6596 | a |
| Sc212m | 0.07 | bc | | | | |
| CML103 | 0.43 | bc | 1.08 | abc | 5173 | ab |
| B73 | 0.21 | c | 0.85 | bcde | 4596 | ab |
| IBM262 | 4.37 | ab | 0.69 | bcde | 4536 | ab |
| B97 | 0.61 | c | 1.75 | a | 3932 | abc |
| Oh7B | 0.51 | bc | | | 3423 | abcd |
| P445…[a] | 0.01 | c | 0.26 | cde | 1675 | abcde |
| Oh43 | 11.01 | a | 0.71 | bcde | 1440 | abcde |
| Il14H | 1.04 | bc | | | | |
| Mp339 | 0.05 | c | 0.64 | cde | 1182 | abcdef |
| Ky21 | 0.69 | bc | 0.91 | abcd | 1046 | abcdef |
| NC350 | 0.67 | bc | | | 889 | abcdef |
| Tx303 | 1.77 | bc | 0.38 | cde | 874 | abcdef |
| MS71 | 0.07 | c | 0.73 | bcde | 705 | bcdef |
| NC358 | 8.78 | a | 0.26 | e | 661 | bcdef |
| NC300 | 0.10 | c | 0.19 | de | 559 | bcdefg |
| IBM54 | 0.41 | bc | | | | |
| M37W | 0.03 | c | 0.20 | e | 541 | bcdefg |
| CML322 | 0.01 | c | 0.28 | cde | 389 | cdefg |
| Ki3 | 0.81 | bc | 0.23 | e | 268 | defg |
| Mp717 | 0.00 | c | | | 264 | bcdefg |
| CML247 | 0.02 | c | 0.37 | cde | 233 | efg |
| CML52 | 0.04 | c | 0.31 | cde | 136 | fg |
| Mp313E | 0.14 | c | 0.31 | cde | 23 | g |

[a] P445… = P445-58-6-4-BBB

[b] Tukey test $\alpha$=0.05

**Table 3.** Pairwise correlations among components of silk resistance to *Aspergillus flavus* in a panel of diverse maize inbreds

| | Field | | *In-vitro* | | |
|---|---|---|---|---|---|
| | Aflatoxin[a] | Colonization[a] | Latent Period[a] | Infection Frequency[a] | Sporulation |
| ---------- Field ---------- | | | | | |
| Colonization[a] | 0.22 | | | | |
| ---------- *In-vitro* ---------- | | | | | |
| Latent Period[a] | 0.23 | -0.17 | | | |
| Infection Frequency[a] | -0.12 | -0.29 | -0.19 | | |
| Sporulation[a] | -0.28 | -0.16 | -0.37 | 0.63** | |
| ---------- Flowering time (Buckler et al. 2009) ---------- | | | | | |
| Days to silk | -0.61* | -0.44 | 0.32 | 0.14 | 0.40 |

[a] The components of resistance studied were: aflatoxin accumulation in ground kernels included here as a control (Aflatoxin), field colonization estimated by qPCR (Colonization), *in-vitro* latent period, *in-vitro* infection frequency and *in-vitro* sporulation

** Significant at $P<0.01$

**Table 4.** Pairwise correlations among components of kernel resistance to *Aspergillus flavus* and other maize characteristics in a panel of diverse maize inbreds

| | Field | | *In-vitro* | |
|---|---|---|---|---|
| | Aflatoxin | Colonization[a] | Colonization[a] | Sporulation |
| ---------- Field ---------- | | | | |
| Colonization | 0.70*** | | | |
| ---------- *In-vitro* ---------- | | | | |
| Colonization | 0.02 | -0.16 | | |
| Sporulation | 0.06 | -0.01 | 0.00 | |
| ---------- Flowering time (Buckler et al. 2009) ---------- | | | | |
| Days to silk | -0.61* | -0.47 | -0.04 | -0.60* |
| ---------- Kernel traits (Flint-Garcia et al. 2009) ---------- | | | | |
| Moisture | -0.03 | -0.02 | 0.33 | -0.53* |
| Protein | -0.45 | -0.21 | -0.38 | 0.39 |
| Fat | -0.25 | -0.17 | 0.03 | 0.27 |
| Fiber | -0.68** | -0.47 | -0.07 | 0.03 |
| Ash | -0.57* | -0.5 | 0.03 | 0.26 |
| Carbohydrate | 0.52* | 0.27 | 0.23 | -0.38 |
| Seed weight | 0.57* | 0.46 | 0.14 | -0.41 |
| % Endosperm | 0.12 | 0.09 | -0.05 | 0.15 |

[a] Colonization estimated by qPCR

*, **, *** Significant at $P<0.05$, 0.01 and 0.001, respectively.

**Table 5.** Components of resistance to Aspergillus Ear Rot: *P*-values by year of study.

| Resistance Component | Silk | | Kernel | | Source of material | Year |
|---|---|---|---|---|---|---|
| | *P* | n | *P* | n | | |
| ----------*In-vitro* inoculation ---------- | | | | | | |
| Latent Period | 0.0129[a] | 19 | ns[b] | 19 | Field NY | 2007 |
| | 0.0003 | 7 | 0.368 | 7 | Green House | 2007 |
| | 0.4269 | 21 | ns[b] | 25 | Field NY | 2008 |
| | 0.0109 | 20 | ns[b] | 26 | Field NY | 2009 |
| Infection Frequency | 0.0001 | 12 | ns[b] | 12 | Field NY | 2007 |
| | 0.0580 | 8 | 0.629 | 8 | Green House | 2007 |
| | 0.1293 | 21 | ns[b] | 25 | Field NY | 2008 |
| | <0.0001 | 20 | ns[b] | 26 | Field NY | 2009 |
| Sporulation | 0.0116 | 17 | <0.0001 | 15 | Field NY | 2007 |
| | 0.0100[a] | 7 | <0.0001 | 7 | Green House | 2007 |
| | 0.4934 | 21 | <0.0001 | 25 | Field NY | 2008 |
| | <0.0001 | 20 | <0.0001 | 26 | Field NY | 2009 |
| Colonization[c] | - | | - | | Field NY | 2007 |
| | <0.0001 | 7 | <0.0001 | 7 | Green House | 2007 |
| | <0.0001 | 19 | <0.0001 | 25 | Field NY | 2008 |
| | - | | <0.0001 | 20 | Field NY | 2009 |
| ----------Field inoculation ---------- | | | | | | |
| Colonization[c] | <0.0001 | 16 | <0.0001 | 20 | Field MS | 2008 |
| | <0.0001 | 24 | <0.0001 | 21 | Field MS | 2009 |
| | <0.0001 | 21 | <0.0001 | 19 | Field MS | 2010 |
| Aflatoxin | - | | <0.0001 | 18 | Field MS | 2008 |
| | - | | <0.0001 | 23 | Field MS | 2009 |
| | - | | 0.0409 | 25 | Field MS | 2010 |

[a] Inbred lines were not significantly different for multiple comparisons using Tukey test.
[b] ns = not significant, no statistical analysis required.
[a] Colonization estimated by qPCR

**Table 6.** Values for various components of silk resistance to *Aspergillus* ear rot after *in-vitro* inoculation by year of study

| Greenhouse | | | | | | Field NY 2007 | | | | | | Field NY 2008 | | | Field NY 2009 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Latent Period (days) | | | Colonization (IC) | | | Infection Frequency (%) | | | Sporulation (%) | | | Colonization (IC) | | | Latent Period (days) | | | Infection Frequency (%) | | | Sporulation (scale) | | |
| Mp339 | 7.60 | a[b] | CML322 | 1.38 | a | Ki3 | 80.43 | a | NC300 | 79.62 | a | CML103 | 2.54 | a | Sc212m | 1.7 | bc | P39 | 100.0 | a | Sc212m | 4.8 | ab |
| Mp313E | 5.23 | b | NC300 | 1.17 | a | M162W | 76.29 | ab | M162W | 46.99 | ab | Oh7B | 2.41 | a | MS71 | 2.2 | abc | Sc212m | 99.4 | ab | NC300 | 4.3 | abc |
| CML322 | 5.02 | b | CML247 | 0.88 | ab | IBM54 | 70.60 | abc | Mo17 | 39.60 | ab | IBM262 | 2.30 | ab | IBM262 | 2.4 | abc | Oh43 | 94.3 | abc | IBM54 | 3.8 | abcd |
| NC300 | 4.91 | b | Mp313E | 0.56 | bc | P445…[a] | 67.52 | abc | CML69 | 38.65 | ab | NC408 | 2.11 | abc | Mp339 | 2.4 | abc | NC300 | 94.0 | abc | M37W | 3.8 | abcd |
| CML52 | 4.85 | b | B73 | 0.39 | bc | B73 | 54.48 | abcd | Ki3 | 33.79 | ab | Mo17 | 1.88 | abc | Tx303 | 2.4 | abc | Tx303 | 93.4 | abc | P39 | 3.8 | abcd |
| B73 | 3.40 | b | CML52 | 0.34 | bc | CML69 | 37.70 | abcd | CML322 | 20.83 | ab | P445-… | 1.73 | abcd | IBM54 | 2.5 | abc | IBM54 | 85.4 | abc | P445… | 3.5 | abcd |
| CML247 | 2.79 | b | Mp339 | 0.18 | c | CML322 | 33.21 | abcd | B73 | 20.46 | ab | Ky21 | 1.44 | abcd | NC300 | 2.5 | abc | Oh7B | 85.4 | abc | Tx303 | 3.5 | abcd |
| | | | | | | NC300 | 32.43 | abcd | Tx303 | 16.95 | ab | Mp339 | 1.37 | abcd | P39 | 2.5 | abc | CML322 | 83.4 | abc | CML322 | 3.3 | abcd |
| | | | | | | IBM262 | 30.00 | abcd | Oh43E | 5.79 | ab | M37W | 1.37 | abcd | NC358 | 2.6 | abc | Il14H | 76.3 | abc | Il14H | 3.3 | abcd |
| | | | | | | Ky21 | 14.64 | abcd | NC358 | 4.98 | ab | Il14H | 1.28 | abcd | CML322 | 2.7 | abc | P445… | 66.4 | abc | B73 | 2.9 | abcd |
| | | | | | | Tx303 | 9.44 | bcd | Ky21 | 4.42 | ab | P39 | 1.26 | abcd | Il14H | 2.7 | abc | Mo17 | 62.9 | abc | Mo17 | 2.8 | abcd |
| | | | | | | M37W | 7.47 | cd | Ms71 | 3.68 | ab | Tx303 | 1.13 | bcd | Oh43 | 2.7 | abc | Ky21 | 59.3 | abc | Oh43 | 2.8 | abcd |
| | | | | | | Oh7B | 0.00 | d | P445… | 2.77 | ab | CML322 | 1.12 | bcd | P445… | 2.8 | abc | B73 | 56.7 | abc | Oh7B | 2.8 | abcd |
| | | | | | | | | | B97 | 1.53 | ab | NC358 | 0.89 | cd | Mo17 | 2.9 | abc | M37W | 52.9 | abc | Ky21 | 2.3 | abcd |
| | | | | | | | | | P39 | 1.53 | ab | B97 | 0.75 | cd | Oh7B | 3.1 | abc | IBM262 | 47.4 | abc | NC358 | 2.3 | abcd |
| | | | | | | | | | M37W | 1.14 | ab | IBM54 | 0.39 | cd | M37W | 3.4 | abc | Mp339 | 38.5 | abc | MS71 | 2.0 | bcd |
| | | | | | | | | | Oh7B | 0.00 | b | B73 | 0.20 | d | B73 | 3.5 | abc | Ki3 | 29.6 | bc | IBM262 | 2.0 | bcd |
| | | | | | | | | | | | | MS71 | 0.11 | d | Ky21 | 3.6 | abc | B97 | 28.2 | bc | B97 | 1.9 | bcd |
| | | | | | | | | | | | | | | | Ki3 | 4.3 | ab | MS71 | 25.0 | c | Mp339 | 1.8 | cd |
| | | | | | | | | | | | | | | | B97 | 4.6 | a | NC358 | 14.9 | c | Ki3 | 1.3 | d |

[a] P445… = P445-58-6-4-BBB
[b] Tukey test α=0.05

**Table 7.** Values for colonization of lines for resistance to silk colonization for field inoculation assays with *Aspergillus flavus* by year of study

| Mississippi 08 | | | Mississippi 09 | | | Mississippi 10 | | |
|---|---|---|---|---|---|---|---|---|
| ---------- Colonization (IC) ---------- | | | | | | | | |
| Oh43 | 22.762 | a[a] | NC358 | 19.555 | a | Oh43 | 13.3 | a |
| IBM262 | 5.238 | b | Il14H | 2.231 | b | IBM262 | 7.9 | ab |
| P39 | 3.389 | bc | Ki3 | 1.049 | b | NC358 | 5.6 | abc |
| NC358 | 2.037 | bc | Oh43 | 1.045 | b | Tx303 | 4.6 | abcd |
| Ki3 | 0.897 | bc | B97 | 0.961 | b | CML103 | 2.7 | abcd |
| B97 | 0.748 | bc | Tx303 | 0.784 | b | NC350 | 1.8 | abcd |
| IBM54 | 0.546 | bc | Oh7B | 0.457 | b | Ky21 | 1.3 | abcd |
| Oh7B | 0.321 | bc | Mo17 | 0.387 | b | Ki3 | 0.5 | bcd |
| B73 | 0.308 | c | IBM262 | 0.336 | b | MS71 | 0.3 | bcd |
| Mp313E | 0.127 | c | B73 | 0.180 | b | B97 | 0.2 | bcd |
| CML69 | 0.109 | c | MS71 | 0.167 | b | Sc212m | 0.2 | bcd |
| MS71 | 0.011 | c | Mp313E | 0.146 | b | Mp717 | 0.1 | bcd |
| CML247 | 0.010 | c | NC300 | 0.126 | b | NC300 | 0.1 | bcd |
| Mp339 | 0.010 | c | CML103 | 0.115 | b | Mo17 | 0.0 | cd |
| M37W | 0.009 | c | Mp339 | 0.113 | b | TBBC3_19 | 0.0 | d |
| CML52 | 0.004 | c | Mp717 | 0.110 | b | M37W | 0.0 | bcd |
| | | | NC350 | 0.105 | b | B73 | 0.0 | cd |
| | | | M37W | 0.077 | b | P445… | 0.0 | cd |
| | | | CML52 | 0.064 | b | CML322 | 0.0 | cd |
| | | | Ky21 | 0.045 | b | Mp339 | 0.0 | bcd |
| | | | CML322 | 0.038 | b | NIL_99 | 0.0 | cd |
| | | | P445… | 0.017 | b | | | |
| | | | IBM54 | 0.012 | b | | | |
| | | | CML247 | 0.007 | b | | | |

[a] Tukey test $\alpha$=0.05

**Table 8.** Pearson correlation (above the diagonal) and Spearman ρ (below the diagonal) for components of silk resistance by year[a]

| | | | Control | *In-vitro* | | | | | | | | Field | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Greenhouse | | 2007 | | 2008 | 2009 | | | 2008 | 2009 | 2010 |
| | | | Aflatoxin[b] | Col[c] | LP | IF | SP | Col | LP | IF | SP | Col | Col | Col |
| Control | | Afla[b] | 1 | -0.15 | 0.11 | -0.21 | -0.21 | 0.42 | -0.17 | 0.23 | 0.10 | 0.31 | 0.04 | 0.20 |
| *In-vitro* | GH | Col[c] | -0.14 | 1 | -0.48 | -0.98 | 0.37 | 0.08 | 0.52 | 0.26 | 0.31 | 0.01 | -0.43 | 0.48 |
| | | LP | -0.04 | -0.18 | 1 | -0.99 | 0.43 | 0.81 | 0.43 | -0.93 | -0.94 | -0.36 | 0.26 | -0.45 |
| | 2007 | IF | -0.16 | -0.50 | -0.50 | 1 | 0.61* | -0.56 | -0.10 | -0.15 | -0.01 | 0.18 | -0.12 | -0.32 |
| | | SP | -0.20 | 0.50 | 0.50 | 0.57 | 1 | -0.26 | -0.25 | 0.18 | 0.18 | -0.09 | -0.07 | -0.08 |
| | 2008 | Col | 0.47 | -0.50 | 1.00*** | -0.52 | -0.22 | 1 | 0.33 | -0.02 | -0.26 | 0.37 | -0.13 | 0.32 |
| | 2009 | LP | -0.14 | 0.80 | 0.40 | -0.05 | -0.25 | 0.38 | 1 | -0.55* | -0.63** | -0.02 | 0.05 | -0.01 |
| | | IF | 0.28 | 0.20 | -1.00*** | -0.06 | 0.12 | -0.01 | -0.35 | 1 | 0.82*** | 0.32 | -0.37 | 0.00 |
| | | SP | 0.02 | 0.20 | -1.00*** | 0.04 | 0.06 | -0.19 | -0.37 | 0.80*** | 1 | -0.04 | -0.25 | -0.27 |
| Field | 2008 | Col | 0.50 | 0.31 | -0.10 | 0.36 | 0.06 | 0.14 | 0.13 | 0.13 | -0.08 | 1 | 0.27 | 0.91*** |
| | 2009 | Col | 0.31 | -0.29 | 0.21 | -0.26 | 0.07 | -0.04 | 0.14 | -0.11 | -0.38 | 0.77** | 1 | 0.43 |
| | 2010 | Col | 0.23 | 0.40 | -0.80 | -0.43 | -0.01 | 0.16 | 0.07 | -0.17 | -0.28 | 0.88** | 0.56** | 1 |

[a] For correlations we only used the components of resistance that presented significant differences for inbred line within a year.
[b] Aflatoxin control values are the least square means from the three years of field inoculation experiments.
[c] The components of resistance evaluated are colonization (Col), latent period (LP), infection frequency (IF) and sporulation (SP).

**Table 9.** Values for various components of developing kernel resistance to *Aspergillus* Ear Rot after *in-vitro* inoculation by year of study

| Greenhouse | | | | | | Field NY 2007 | | | Field NY 2008 | | | | | | Field NY 2009 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sporulation (%) | | | Colonization (IC) | | | Sporulation (%) | | | Sporulation (%) | | | Colonization (IC) | | | Sporulation (%) | | | Colonization (IC) | | |
| CML247 | 76.0 | a[b] | CML247 | 7.01 | a | NC358 | 67.2 | a | NC350 | 85.8 | a | IBM262 | 5.35 | a | Sc212m | 80.3 | a | Sc212m | 9.24 | a |
| Mp339 | 7.9 | b | B73 | 2.70 | a | Mo17 | 62.8 | a | Oh7B | 83.4 | a | Tx303 | 3.30 | ab | Il14H | 77.5 | a | IBM262 | 8.80 | ab |
| NC300 | 4.2 | bc | Mp339 | 0.74 | b | IBM54 | 61.7 | a | P445…[a] | 78.1 | ab | Ky21 | 3.06 | abc | MS71 | 67.4 | abc | M37W | 6.00 | abc |
| Mp313E | 3.4 | bc | NC300 | 0.22 | b | B73 | 57.1 | a | P39 | 77.9 | ab | Il14H | 2.05 | abcd | IBM262 | 65.7 | abcd | Ki3 | 5.62 | abc |
| B73 | 2.9 | bc | CML322 | 0.09 | b | M162W | 53.3 | abc | Ms71 | 73.6 | ab | Mp339 | 1.80 | abcd | M37W | 63.8 | abcde | Tx303 | 5.23 | abc |
| CML52 | 1.9 | bc | Mp313E | 0.08 | b | B97 | 52.6 | ab | Mo17 | 72.1 | ab | M37W | 1.33 | abcd | CML322 | 60.5 | abcdefg | Oh7B | 4.59 | abc |
| CML322 | 0.7 | c | CML52 | 0.08 | b | IBM262 | 39.5 | abc | CML322 | 68.2 | ab | CML103 | 1.31 | abcd | B73 | 59.3 | abcdef | Mp339 | 3.86 | abc |
| | | | | | | NC300 | 38.2 | abcd | B73 | 67.2 | ab | Mo17 | 1.18 | abcd | Mp339 | 57.5 | abcdefg | NC358 | 3.07 | abc |
| | | | | | | Ky21 | 36.9 | abcd | IBM262 | 66.4 | ab | CML247 | 1.02 | abcd | NC358 | 52.9 | abcdefgh | MS71 | 2.98 | abc |
| | | | | | | Oh7B | 27.3 | bcd | IBM54 | 63.7 | abc | P39 | 0.95 | abcd | IBM54 | 50.4 | abcdefgh | B97 | 2.86 | abc |
| | | | | | | CML69 | 25.9 | bcd | Sc212m | 61.6 | abc | CML322 | 0.95 | abcd | P445… | 37.5 | bcdefghi | Ky21 | 2.59 | abc |
| | | | | | | CML322 | 22.0 | cd | CML69 | 56.0 | abc | B73 | 0.66 | bcd | B97 | 35.2 | bcdefghi | P39 | 2.47 | abc |
| | | | | | | Oh43E | 18.5 | cd | M37W | 50.6 | abc | P445… | 0.51 | abcd | Ky21 | 30.4 | bcdefghi | Oh43 | 2.42 | abc |
| | | | | | | Tx303 | 11.5 | d | NC358 | 50.0 | abc | IBM54 | 0.43 | abcd | CML103 | 28.8 | bcdefghi | CML322 | 2.07 | abc |
| | | | | | | M37W | 10.2 | d | Il14H | 49.9 | abc | B97 | 0.29 | cd | Oh7B | 28.2 | cdefghi | P445… | 1.81 | abc |
| | | | | | | | | | CML103 | 46.5 | abc | CML69 | 0.23 | cd | Oh43 | 27.6 | cdefghi | Il14H | 1.74 | abc |
| | | | | | | | | | B97 | 39.8 | bc | MS71 | 0.16 | d | Ki3 | 26.6 | defghi | B73 | 1.71 | bc |
| | | | | | | | | | Mp339 | 33.8 | bc | Sc212m | 0.09 | cd | CML69 | 24.4 | bcdefghi | Mo17 | 1.58 | c |
| | | | | | | | | | Tx303 | 27.0 | bc | Oh7B | 0.06 | d | NC300 | 20.9 | fghi | NC350 | 1.13 | c |
| | | | | | | | | | Ky21 | 26.8 | bc | NC408 | 0.05 | d | Mo17 | 19.0 | ghi | NC300 | 0.86 | c |
| | | | | | | | | | Tx303 | 17.9 | c | NC358 | 0.05 | d | P39 | 13.2 | hi | | | |
| | | | | | | | | | CML247 | 14.6 | c | | | | Tx303 | 12.2 | i | | | |
| | | | | | | | | | | | | | | | NC350 | 9.8 | i | | | |

[a] P445… = P445-58-6-4-BBB

[b] Tukey test α=0.05

**Table 10.** Values for components of kernel resistance for field inoculation assays with *Aspergillus flavus* by year of study

| Mississippi 08 | | | | | | Mississippi 09 | | | | | | Mississippi 10 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Aflatoxin (ng/g) | | | Colonization (IC) | | | Aflatoxin (ng/g) | | | Colonization (IC) | | | Aflatoxin (ng/g) | | | Colonization (IC) | | |
| CML103 | 22735 | a[b] | CML103 | 1.183 | a | Mo17 | 13379 | a | Il14H | 2.18 | a | Oh7B | 6252 | a | NIL_99 | 4.91 | ab |
| B73 | 13917 | ab | Mo17 | 0.850 | ab | IBM262 | 7376 | ab | B97 | 1.98 | a | Mp339 | 3473 | ab | B97 | 4.68 | a |
| Mo17 | 11982 | ab | B73 | 0.668 | abc | CML103 | 4244 | ab | IBM262 | 1.08 | ab | B73 | 2969 | ab | Ky21 | 4.17 | ab |
| B97 | 10118 | abc | B97 | 0.391 | bcd | Oh43 | 4045 | ab | MS71 | 0.85 | abc | Sc212m | 2599 | ab | Mp339 | 3.59 | abc |
| IBM262 | 6283 | abcd | MS71 | 0.328 | bcd | B97 | 3656 | ab | CML103 | 0.79 | abcd | Ky21 | 2244 | ab | Oh43 | 2.88 | abc |
| MS71 | 3689 | abcde | Oh43 | 0.311 | bcd | B73 | 2123 | abc | Oh43 | 0.65 | bcd | IBM262 | 2014 | ab | B73 | 2.45 | abc |
| Oh7B | 3483 | abcde | Oh7B | 0.230 | cd | Mp339 | 1353 | abcd | Tx303 | 0.58 | bcd | Mo17 | 1790 | ab | TBBC3_19 | 2.12 | abc |
| IBM54 | 2790 | bcde | IBM54 | 0.119 | d | P445…[a] | 1219 | abcd | Mp339 | 0.55 | bcd | B97 | 1643 | ab | Mo17 | 1.76 | abc |
| Oh43 | 2542 | bcde | NC358 | 0.113 | d | MS71 | 1101 | abcd | B73 | 0.45 | bcd | CML103 | 1434 | ab | CML247 | 1.71 | abc |
| Ki3 | 1739 | bcde | IBM262 | 0.099 | d | Tx303 | 1057 | abcd | CML322 | 0.39 | bcd | TBBC3_19 | 1360 | ab | CML103 | 1.26 | abc |
| Tx303 | 1333 | cdef | Ky21 | 0.081 | d | Il14H | 637 | abcde | M37W | 0.33 | bcd | P445… | 1312 | ab | Tx303 | 1.13 | abc |
| Ky21 | 1056 | defg | CML69 | 0.069 | cd | NC358 | 603 | bcd | Ky21 | 0.30 | bcd | NC350 | 777 | ab | P445… | 0.99 | abc |
| Mp339 | 886 | defg | Tx303 | 0.058 | d | M37W | 566 | abcd | NC358 | 0.28 | bcd | NIL_99 | 737 | ab | NC350 | 0.78 | bc |
| NC358 | 854 | defg | Ki3 | 0.055 | d | NC300 | 494 | abcd | Ki3 | 0.24 | bcd | M37W | 654 | ab | CML322 | 0.75 | bc |
| M37W | 622 | efgh | Mp339 | 0.054 | d | Ky21 | 483 | bcd | P445… | 0.21 | cd | NC358 | 560 | ab | NC300 | 0.36 | abc |
| CML247 | 172 | fgh | M37W | 0.018 | d | NC350 | 355 | abcde | NC300 | 0.20 | bcd | Tx303 | 520 | ab | M37W | 0.28 | bc |
| Mp313E | 126 | gh | CML52 | 0.004 | d | CML322 | 181 | cde | Mp715 | 0.11 | d | CML322 | 476 | ab | Mp717 | 0.27 | c |
| CML52 | 94 | h | Mp313E | 0.004 | d | CML247 | 155 | cde | Mp313E | 0.11 | bcd | CML247 | 473 | ab | Ki3 | 0.25 | bc |
| | | | NC300 | 0.003 | d | CML52 | 66 | def | CML52 | 0.09 | bcd | Mp717 | 467 | ab | NC358 | 0.18 | c |
| | | | CML247 | 0.001 | d | Mp717 | 34 | def | CML247 | 0.00 | cd | NC300 | 447 | ab | | | |
| | | | | | | Ki3 | 7 | ef | | | | Ki3 | 436 | ab | | | |
| | | | | | | Mp715 | 7 | ef | | | | CML69 | 385 | ab | | | |
| | | | | | | Mp313E | 1 | f | | | | CML52 | 348 | ab | | | |
| | | | | | | | | | | | | Oh43 | 290 | ab | | | |
| | | | | | | | | | | | | MS71 | 86 | b | | | |

[a] P445… = P445-58-6-4-BBB

[b] Tukey test α=0.05

**Table 11.** Pearson correlation (above the diagonal) and Spearman ρ (below the diagonal) for components of kernel resistance by year[a]

| | | | Control | *in-vitro* | | | | | | | Field | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Greenhouse | | 2007 | 2008 | | 2009 | | 2008 | | 2009 | | 2010 | |
| | | | Aflatoxin[b] | SP | Col | SP | SP | Col | SP | Col | Aflatoxin | Col | Aflatoxin | Col | Aflatoxin | Col |
| Control | | Afla[b] | 1 | -0.08 | 0.36 | 0.51 | 0.43 | 0.06 | -0.08 | -0.02 | 0.90*** | 0.70** | 0.92*** | 0.69** | 0.67*** | 0.56* |
| In-vitro | GH | SP | 0.11 | 1 | 0.84* | 0.68 | -0.90 | 0.05 | -0.22 | 0.37 | -0.25 | -0.25 | 0.03 | -0.51 | -0.16 | 0.18 |
| | | Col | 0.61 | 0.64 | 1 | 0.91 | -0.63 | -0.31 | 0.39 | 0.12 | 0.33 | 0.35 | 0.36 | -0.17 | 0.23 | 0.44 |
| | 2007 | SP | 0.60 | 0.50 | 1.00*** | 1 | 0.22 | -0.34 | 0.09 | -0.46 | 0.50 | 0.56 | 0.48 | 0.19 | 0.32 | 0.28 |
| | 2008 | SP | 0.28 | -1.00*** | -0.80 | 0.19 | 1 | -0.40 | 0.08 | -0.15 | 0.59* | 0.37 | 0.40 | 0.14 | 0.11 | -0.34 |
| | | Col | 0.10 | 0.60 | 0.00 | -0.37 | -0.45* | 1 | -0.05 | 0.17 | -0.09 | -0.16 | 0.15 | 0.13 | 0.15 | 0.26 |
| | 2009 | SP | -0.09 | -0.80 | -0.20 | -0.02 | 0.04 | -0.02 | 1 | 0.37 | -0.16 | -0.11 | 0.02 | 0.27 | 0.08 | -0.05 |
| | | Col | -0.18 | 0.20 | 0.00 | -0.40 | -0.24 | 0.00 | 0.37 | 1 | -0.39 | -0.46 | -0.07 | 0.06 | 0.19 | -0.09 |
| Field | 2008 | Afla | 0.90*** | 0.30 | 0.70 | 0.37* | 0.50 | -0.06 | -0.12 | -0.47 | 1 | 0.81*** | 0.74** | 0.77*** | 0.38 | 0.26 |
| | | Col | 0.85*** | -0.54 | -0.09 | 0.68 | 0.56* | -0.33 | 0.05 | -0.41 | 0.92*** | 1 | 0.59* | 0.52* | 0.28 | 0.26 |
| | 2009 | Afla | 0.92*** | 0.11 | 0.61 | 0.55 | 0.33 | 0.05 | 0.04 | 0.03 | 0.81*** | 0.79*** | 1 | 0.58** | 0.44 | 0.56* |
| | | Col | 0.73*** | -0.14 | 0.18 | 0.03 | 0.05 | 0.24 | 0.34 | 0.15 | 0.8*** | 0.78*** | 0.78*** | 1 | 0.22 | 0.53* |
| | 2010 | Afla | 0.69*** | 0.20 | 0.49 | 0.33 | 0.10 | 0.11 | 0.22 | 0.16 | 0.31 | 0.27 | 0.51* | 0.28 | 1 | 0.62** |
| | | Col | 0.63** | 0.30 | 0.50 | 0.10 | -0.40 | 0.18 | -0.10 | -0.12 | 0.27 | 0.31 | 0.57* | 0.52 | 0.58* | 1 |

[a] For correlations we only used the components of resistance that presented significant differences for inbred lines within a year.
[b] Aflatoxin field mean are the least square means from the three years of field inoculation experiments.
[c] The components of resistance evaluated are colonization (Col), aflatoxin accumulation (Afla) and sporulation (SP).

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CHAPTER 4

# MAPPING QTL AFFECTING MULTIPLE COMPONENTS OF MAIZE RESISTANCE TO *Aspergillus flavus*

## *Introduction*

Colonization of maize by the fungus *Aspergillus flavus* can result in accumulation of various mycotoxins. The most dangerous mycotoxins are aflatoxins, which are secondary metabolites that have extremely deleterious effects on humans and animals. Several authors have reported maize lines with genetic resistance to aflatoxin accumulation (1, 5, 19, 21). Resistance to aflatoxin accumulation is quantitative and highly influenced by the environment (Chapter 3) (1, 4, 5, 20). Previously, in a panel of diverse maize lines, we found that there is resistance to silk and kernel colonization by *A. flavus* (Chapter 3).

Broad sense heritability for resistance to aflatoxin accumulation has been calculated in several populations. Reports range from no heritability in the B73 x Oh516 population (4) to 74% for the B73 x M182 population (8). QTL for resistance to aflatoxin accumulation have been reported in several studies (2, 4, 11, 16-18). Because of the strong variation, few QTL were identified over multiple years, so analyses have typically been conducted on data from each year separately. The

reported QTLs encompass regions from 8 to 38 cM on a consensus genetic map (Chapter 5).

In this study we conducted QTL mapping in a population of recombinant inbred lines derived from a cross between B73 and CML322. Previous reports indicated that CML322, a tropical line with white endosperm, was among the most resistant lines to aflatoxin accumulation (Chapters 2, 3) (1, 10). The population is part of the nested association mapping (NAM) population and has been densely genotyped (9). In order to identify novel QTL for resistance to *A. flavus*, we evaluated five components of resistance to silk and kernel infection for three years, using *in-vitro* and field inoculation. We hypothesized that resistance to the various components of resistance would map to different regions of the genome. To confirm QTL in this population, we developed near-isogenic lines (NILs) and tested them in one year of *in-vitro* inoculation assays. In addition, we used two NILs developed by Syngenta.

### *Materials and Methods*

**Mapping Population.** The B73 x CML322 population from the NAM project (9) was used for QTL mapping. The population is composed of recombinant inbred lines (RILs) at the S5 level. Genotyping data for the whole population for 1200 SNP markers is publicly available (3, 9). In 2007, 2008 and 2009, 120, 162 and 153 lines of the population, respectively, were planted at Cornell's Robert Musgrave Research Farm in Aurora, NY. Each line was planted in a single row. Silk or developing kernel tissue was collected and transported to the lab for *in-vitro* inoculations, as explained

below. In 2008, 2009 and 2010, 95, 148 and 179 lines were planted at the R. R. Foil Plant Science Research Center at Mississippi State University, MS. Lines were planted in three blocks in a randomized complete blocks design and field inoculated as explained below.

**Fungal Isolate and Inoculation Procedures.** Inocula for *in-vitro* and field inoculations were prepared as explained previously (Chapter 3). Briefly, we used $1x10^7$ conidia per milliliter of *A. flavus* isolate NRRL 3357 for *in-vitro* inoculations and $3x10^8$ conidia per milliliter of the same isolate for field inoculations. In the field, maize ears were inoculated directly into the kernels and in the silk channel using a tree-marking gun.

QTL mapping was conducted for five components of resistance, here referred to as traits. Silk infection frequency, sporulation and developing kernel sporulation were evaluated with *in-vitro* inoculations, while silk colonization and aflatoxin accumulation at maturity were evaluated from field-inoculated plants. *In-vitro* inoculations were conducted on tissue grown in the field in NY, as explained previously. Shoots were covered before emergence and one day after sib or self pollination, silk tissue was transported to the lab and five silks in 2007 or ten silks in 2008 and 2009 were inoculated with one drop of a conidial suspension placed at the tip of the silk. Infection frequency and sporulation were evaluated seven days after inoculation. Each year, tissue from four different plants was analyzed as four replications. Trays were arranged in a block design in the growth chamber. For

developing kernel assays, ears were collected from the field three weeks after pollination and transported to the lab. Five developing kernels were carefully excised and placed in petri plates in a humid chamber as described previously (Chapter 3). Seven days after inoculation, the percentage area covered by sporulation was rated with the help of a dissecting microscope.

Field inoculation was conducted in MS as explained previously (Chapter 3). Seven days after inoculation, silk materials were collected and frozen until DNA extraction and qPCR was conducted to estimate the levels of fungal colonization of the silk channel (Chapter 2, 3)(10). For aflatoxin determination, ears were harvested at maturity, dried and ground. Fifty grams of each subsample were used for aflatoxin quantification using the VICAM AflaTest (Watertown, MA).

**Statistical Analysis.** Phenotypic data were analyzed for each year independently and for the three years of data together. JMP V 8.0 (SAS Institute, Cary, NC) was used for calculation of the least square means (LSM) and heritability. For the statistical analysis and LSM estimation, replicates were considered random factors while lines of the population were considered fixed factors. In the overall analysis, years were included in the model as a random factor. For the estimation of broad sense heritability ($H^2$), all the factors in the model were considered random (12).

**QTL Mapping.** Least squares means for the five traits were used for QTL mapping. In addition to the combined data for three years, data from each year were

also used for mapping. As a result, QTL mapping was conducted on 20 data sets. QTL mapping was conducted with a stepwise regression approach as described by Buckler et al. (3) and implemented using GLMSELECT in SAS V 9.2 (SAS Institute, Cary, NC). The cut-off value (threshold of significance) for entry of markers in the model was 0.001, and markers were retained if their p-values were less than 0.001 (3). Allelic effects for each marker (QTL) were obtained from the GLMSELECT output. Confidence intervals for each QTL were created by successively adding flanking markers to the model on each side of the selected marker until a marker was found not to be associated with the trait at $\alpha$=0.05 (7).

**Near Isogenic Line Development and Testing.** Preliminary data analysis in 2007 and 2008 indicated the presence of QTL for resistance to aflatoxin accumulation, developing kernel sporulation, and silk sporulation in maize bins 4.08, 6.07 and 8.06 respectively. These QTL were designated qbAFL4.08, qbKSP6.07 and qbSSP8.05. QTL qbKSP6.07 was identified in 2008 and 2009 data but with lower cut-off thresholds. Near isogenic lines (NILs) were developed using the heterogeneous inbred family approach (13). Sixteen B73 x CML322 $F_2S_5$ lines that were heterozygous at eight SNP markers at or near the significant QTL marker were identified and self pollinated for two generations in 2009 in NY and in a winter nursery in Puerto Rico. Six hundred and thirty four lines were planted in NY in 2010. These lines corresponded to eight families for qbAFL4.08, four families for qbKSP6.07 and nine families for qbSSP8.05.

Each of the 634 lines were genotyped by allele specific PCR, using KASPar chemistry and protocols (KBioscience, Hoddesdon, Hertfordshire, UK) and using dried CTAB extracted DNA (Jamann et al. unpublished). Two to four lines that differed at the appropriate SNP marker for each family were identified and inoculated *in-vitro* as indicated above in 2010. Four components of resistance were evaluated on these lines: developing kernel sporulation, silk infection frequency, silk sporulation and silk latent period.

In addition, two NILs produced by Syngenta that had fixed introgressions from lines CML103 and Tx303 at markers within qbAFL4.08 were identified. The pedigree of these lines was $(B73^6/CML103)S_3$ and $(B73^6/Tx303)S_3$ and they had been designated NIL10 and NIL99. NIL10 was tested for *in-vitro* components of resistance only in 2010 while NIL99 was tested *in-vitro* in 2009 and 2010 and with field inoculations in 2010.

***Results***

**QTL Mapping.** At least one QTL was found for every trait analyzed (Table 1). Means for three years of data for the parental lines were significantly different for aflatoxin accumulation (Figure 1). Transgressive segregation was observed for all traits. In three years of phenotypic evaluations, no variance was found to be due to genotypes (RILs) for silk infection frequency and silk sporulation on *in-vitro* inoculation assays. Estimates of $H^2$ for *in-vitro* sporulation on developing kernels and

field colonization in silk tissue were low while moderate $H^2$ was found for aflatoxin accumulation in field inoculation assays (Table 2).

Thirteen QTLs spanning regions from 12 to 60 cM on the B73 x CML322 genetic map were identified for the 20 data sets analyzed (Table 1). One QTL was identified for silk infection frequency and another for silk sporulation despite the fact that no heritability was detected for these two traits. QTLs for *in-vitro* sporulation on developing kernels were identified in the 2008 data as well as in the combined analysis across years (qbKSP8.02 and qaKSP8.03). Similarly, for field colonization of silks, one QTL was found on chromosome 4 in the combined three-year data set as well as the 2010 data. Four aflatoxin-accumulation QTL were identified on chromosomes 4, 7 and 10. The QTL on chromosome 10 was significant in the combined three-year data set as well as in the 2010 data.

Three QTL for resistance in field inoculation assays co-localized to chromosome 4 bins 4.08 and 4.09 while other three QTL co-localized to chromosome 10 bins10.06 and 10.07 (Fig 2). In addition, four QTL for *in-vitro* inoculation assays co-localized to chromosome 8 bins 8.02 to 8.05. Single QTLs for aflatoxin accumulation and developing kernel sporulation were found on chromosomes 7 and 9. A QTL for silk infection frequency was found on chromosome 4.

**Near Isogenic Lines.** Twenty-two families of heterogeneous inbred lines with opposite alleles at the loci of interest were inoculated *in-vitro* in 2010. Each one of

these families has the target chromosome segment in a different genetic background that represents a different combination of parental alleles. Significant differences were found in five of the eight families developed for qbAFL4.08. For sporulation on developing kernels, three families had lines that were significantly different (Table 3). In two of the families (C and F), the CML322 allele was significantly more resistant but in the third family (G) the B73 allele was more resistant (Table 3). For family E, there were significant differences among lines for silk infection frequency and for silk sporulation (Tables 4 and 5).

Significant differences were found within two out of five families developed for qbKSP6.07. For family D, the B73 allele conferred resistance (as expected) to sporulation on developing kernels. There were also significant differences in family A for latent period in silk (Table 6). Significant differences were found in four of the nine families developed for qbSSP8.05. In three families (D, E and G), there were differences for sporulation on developing kernels. For two of them, the B73 allele was more resistant while for the other family the CML322 allele was more resistant (Table 3). Finally, in one family (I), there were significant differences for sporulation on silks were the B73 allele was more resistant than the CML322 allele (opposite from expected) (Table 5).

The Syngenta line NIL99 was significantly different from B73 for *in-vitro* developing kernel sporulation, silk sporulation and silk latent period in 2009 and for silk latent period in 2010 (Figure 3). This line was also evaluated with field

inoculations in 2010, where it accumulated lower levels of aflatoxin than B73 but this difference was not statistically significant (Figure 3E). There were no significant differences for *in-vitro* developing kernel sporulation, silk latent period, silk sporulation or silk infection frequency between NIL10 and the recurrent parent B73 (data not shown).

***Discussion***

Because of the strong environmental effect reported previously (Chapter 3), for this study, QTL analysis was conducted on a per year basis and on a combined basis. The parental lines (B73 and CML322) have been reported to be significantly different only for aflatoxin accumulation (1) (Chapter 3). In this study, we did not find differences for other silk or kernel traits. However, the presence of transgressive segregation indicates that there are combinations of genes in the population that could allow us to map QTL for resistance to these traits. This also suggests that recurrent selection for aflatoxin resistance could lead to the accumulation of resistance factors.

Heritabilities allow for comparisons of traits within and across populations (14). Broad sense heritability for aflatoxin accumulation has been reported to range from non-existent on the B73xOh516 population (4) to 74% in the B73 x MI82 population (8). Our $H^2$ of 63% for aflatoxin is towards the high range of previously reported heritability and indicates that selection for resistance is possible for this population. The low $H^2$ found for silk colonization (11%) and kernel sporulation (14%) are lower than reported values of 21% and 84% for BGYF in B73 x Oh516 and

B73 x MI82, respectively (4, 8). $H^2$ for ear rot ratings has ranged from 11% for the B73 x Oh516 population to 62% for the B73 x MI82 population (4, 8). Considering the added cost and time of conducting *in-vitro* inoculations or evaluating silk colonization by qPCR compared to ear rot ratings, it seems clear that the former traits are not useful for breeding purposes, at least on our B73 x CML322 population. However, the objective of this study was to discover novel QTL associated with resistance to silk colonization and sporulation on kernels. Our two other *in-vitro* silk traits, disappointingly, had heritabilities of zero. Busboom and White (4) found the same situation on their B73 x Oh516 population for aflatoxin accumulation while moderate $H^2$ was reported for other populations (6, 8, 11, 15).

QTL for resistance to aflatoxin accumulation have been detected on all maize chromosomes except for chromosome 9 (Table 4 of Chapter 5). In this study we found a QTL on chromosome 9 for sporulation on developing kernels in one year of data. The largest number of QTL reported for aflatoxin accumulation for any chromosome is 11 on chromosome 4 (Chapter 5). In this study we found that four out of 13 QTL were located on chromosome 4. In the field inoculation assays, we also found a QTL for resistance to aflatoxin accumulation on chromosome 4. This chromosome also harbored a QTL for silk colonization that was significant in one year of data as well as in the combined data set. From *in-vitro* inoculation assays, we found a QTL for silk infection frequency on chromosome 4. The repeated localization of QTLs for resistance to aflatoxin accumulation and other components of resistance

in multiple populations and by various authors make this chromosome a high priority target for the dissection of this trait.

Four QTL were found on chromosome 8. Prior to this, only one QTL for resistance to aflatoxin had been reported on this chromosome (Table 4 in Chapter 5). Co-localization of QTL for field resistance of silk and kernel traits on chromosome 4, as well as the co-localization of QTL for *in-vitro* inoculation for silk and kernel traits on chromosome 10, suggests some degree of genetic correlation for these traits. No correlation for silk and kernel traits was found among diverse inbred lines in a previous study (Chapter 3). This discrepancy might be due to the small effect of each of these QTL. In addition, considerable environmental variance makes differences due to these QTL difficult to detect with the power used in our experiments.

The $R^2$ of QTLs for resistance to aflatoxin accumulation in previous QTL mapping experiments is lower than 0.15 for most previously reported QTL (Table 4 in Chapter 5). One exception is a QTL found in the Mp313E x B73 population on chromosome 4 and another in the Mp313E x Va35 population on chromosome 1 with reported $R^2$ of 0.21 and 0.22, respectively. Compared to these effects, several QTLs in our population are larger, especially those for aflatoxin accumulation with $R^2$ values that range from 0.25 to 0.41. There are substantial differences between the methods for QTL mapping between our experiment and those of the previously reported mapping experiments. The previous QTL mapping experiments used composite interval mapping while in our experiment we used stepwise regression applied on a

denser genetic map. The heritability of aflatoxin accumulation in our population was higher than expected and this factor, along with high density of our map, could produce a more precise location of the QTL and perhaps also a higher $R^2$.

Chromosome 4 was the target for the selection of two near isogenic lines from the Syngenta NILs. In this study, we found the largest effect QTL qbAFL4.08 in maize bin 4.08. NIL10 and NIL99 had introgressions in this region. NIL99 was significantly more resistant than B73 in two years for *in-vitro* and field inoculation studies. To our knowledge, this is the first time that a QTL for resistance to aflatoxin accumulation has been confirmed with near isogenic lines. In addition, we generated our own NILs from the B73 x CML322 population. In one year of data, two families designed for qbAFL4.08 (C and F), showed the CML322 allele, to be more resistant than the B73 allele, as expected. No aflatoxin accumulation data on these families has been gathered yet but this experiment is planned for 2011. In addition, for family D of NILs targeting qbKSP6.07, the B73 allele was more resistant than the CML322 allele as expected. This family has also been included in tests for 2011.

Overall, we have thoroughly studied the inheritance of resistance to *A. flavus* in the NAM B73 x CML322 population. We found moderate levels of heritability for aflatoxin accumulation and low levels for silk colonization and *in-vitro* sporulation. We have found QTL for resistance to silk-related traits. We described a medium-effect QTL on chromosome 4, bin 4.08 and confirmed this QTL in a NIL developed by Syngenta. It would be important to further characterize these QTLs as a means to

better understand the *A. flavus* – maize interaction, and to assess its use in breeding resistant materials.

**Table 1.** QTL identified using a step wise regression approach in the B73 x CML322 population for five traits phenotyped over three years.

| QTL | Tissue | Trait | Marker | Bin | Position (cM) | Position RefGen_V2 | Effect | *P* | CI start | CI end | QTL interval (cM) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ---------- *In-vitro* inoculation ---------- | | | | | | | | | | | |
| qbSIF4.05 | Silk | Infection Frequency (2009) | PZA00445 | 4.05 | 55.2 | 49,917,660 | -0.14 | 0.00049 | PZA02358 | PZA00453 | 45 |
| qbKSP8.02 | Dev. Kernels | Sporulation (2009) | PZA02454 | 8.02 | 42.0 | 18,215,366 | 0.08 | 0.00002 | PZA03178 | PZB02155 | 37 |
| qaKSP8.03 | Dev. Kernels | Sporulation (3 years) | PHM3978 | 8.03 | 57.5 | 101,178,563 | 0.06 | 0.00005 | PZA03178 | PZA00118 | 38 |
| qbSSP8.05 | Silk | Sporulation (2008) | PZA00429 | 8.05 | 74.3 | 145,842,587 | -0.18 | 0.00010 | PZA01470 | PZA00505 | 60 |
| qbKSP9.07 | Dev. Kernels | Sporulation (2008) | PZA03573 | 9.07 | 114.8 | 154,462,461 | 0.07 | 0.00060 | PZA00708 | PZA03573 | 12 |
| ---------- Field inoculation ---------- | | | | | | | | | | | |
| qbAFL4.08 | Mat. Kernels | Aflatoxin (2008) | PHM3637 | 4.08 | 92.7 | 180,672,091 | -0.41 | 0.00005 | PZA00453 | PZA00694 | 40 |
| qaSCO4.09 | Silk | Colonization (3 years) | PZA03155 | 4.09 | 112.2 | 216,608,367 | 0.02 | 0.00008 | PZA01187 | PHM2100 | 38 |
| qbSCO4.09 | Silk | Colonization (2010) | PZA00878 | 4.09 | 112.5 | 220,606,809 | 0.03 | 0.00025 | PZA03275 | PZA03322 | 43 |
| qbAFL7.04 | Mat. kernels | Aflatoxin (2009) | PZA00795 | 7.04 | 105.2 | 165,102,982 | 0.28 | 0.00068 | PZA03176 | PZA00695 | 31 |
| qbSCO8.04 | Silk | Colonization (2010) | PHM3993 | 8.04 | 64.2 | 120,061,120 | -0.03 | 0.00018 | PZA01186 | PZA00951 | 33 |
| qbAFL10.06 | Mat. kernels | Aflatoxin (2010) | PZA03607 | 10.06 | 75.4 | 142,189,643 | -0.29 | 0.00040 | PZA02320 | PZA00062 | 38 |
| qaAFL10.07 | Mat. kernels | Aflatoxin (3 years) | PZA00130 | 10.07 | 80.8 | 143,674,115 | -0.25 | 0.00015 | PZA02663 | PZA02527 | 37 |
| qaSCO10.07 | Silk | Colonization (3 years) | PZA02578 | 10.07 | 91.2 | 147,014,677 | -0.02 | 0.00216 | PZA01073 | PZA02527 | 19 |

**Table 2.** Components of variance used for the calculation of broad sense heritability ($H^2$) on a line mean basis for five traits phenotyped in the B73 x CML322 population for three years.

| | *In-vitro* Inoculation | | | Field Inoculation | |
|---|---|---|---|---|---|
| | Silk | | DvK | Silk | Kernel |
| | Infection Frequency | Sporulation | Sporulation | Colonization | Aflatoxin |
| Pedigree variance | 0 | 0 | 0.0066 | 0.0006 | 0.47 |
| Year variance | 0.11 | 0.16 | 0.0023 | 0.0002 | 0.28 |
| Error variance | 0.17 | 0.24 | 0.12 | 0.014 | 0.56 |
| $H^2$ | | | 0.14 | 0.11 | 0.63 |

**Table 3**. Near isogenic line families with significant differences for developing kernel sporulation (seven out of 22 tested).

| Line | Sporulation (%) | | Genotype at locus (QTL) PHM3637 (qbAFL4.08) | PHM7922 (qbKSP6.07) | PZA02011 (qbSSP8.05) |
|---|---|---|---|---|---|
| ---------- Parents ---------- | | | | | |
| B73 | | | AA | AA | AA |
| CML322 | | | CC | CC | GG |
| ---------- qbAFL4.08 family C* ---------- | | | | | |
| 10SH0121 | 64 | a | AA | | |
| 10SH0119 | 45 | b | AA | | |
| 10SH0116 | 37 | b | CC | | |
| ---------- qbAFL4.08 family F* ---------- | | | | | |
| 10SH0565 | 91 | a | AA | | |
| 10SH0560 | 64 | b | CC | | |
| 10SH0562 | 54 | b | CC | | |
| ---------- qbAFL4.08 family G ---------- | | | | | |
| 10SH0580 | 79 | a | CC | | |
| 10SH0571 | 47 | b | AA | | |
| ---------- qbKSP6.07 family D* ---------- | | | | | |
| 10SH0428 | 80 | a | | CC | |
| 10SH0429 | 74 | a | | CC | |
| 10SH0431 | 67 | ab | | AA | |
| 10SH0432 | 36 | b | | AA | |
| ---------- qbSSP8.05 family D ---------- | | | | | |
| 10SH0166 | 93 | a | | | AA |
| 10SH0160 | 79 | a | | | GG |
| 10SH0165 | 58 | b | | | AA |
| ---------- qbSSP8.05 family E ---------- | | | | | |
| 10SH0181 | 87 | a | | | AA |
| 10SH0187 | 67 | b | | | GG |
| ---------- qbSSP8.05 family G ---------- | | | | | |
| 10SH0547 | 57 | a | | | AA |
| 10SH0544 | 44 | ab | | | GG |
| 10SH0546 | 23 | b | | | AA |

* Further characterization is planned in the summer of 2011

**Table 4.** Heterogeneous inbred families with significant differences for silk infection frequency (one out of 22 tested).

| Line | Infection frequency (%) | | Genotype PHM3637 (qbAFL4.08) |
|---|---|---|---|
| ---------- Parents ---------- | | | |
| B73 | | | AA |
| CML322 | | | CC |
| ---------- qbAFL4.08 family E ---------- | | | |
| 10SH0223 | 99.83 | a | CC |
| 10SH0224 | 38.46 | b | AA |

**Table 5.** Heterogeneous inbred families with significant differences for silk sporulation (two out of 22 tested).

| Line | Sporulation (scale) | | Genotype PHM3637 (qbAFL4.08) | PZA02011 (qbSSP8.05) |
|---|---|---|---|---|
| ---------- Parents ---------- | | | | |
| B73 | | | AA | AA |
| CML322 | | | CC | GG |
| ---------- qbAFL4.08 family E ---------- | | | | |
| 10SH0223 | 3.28 | a | CC | |
| 10SH0224 | 1.82 | b | AA | |
| ---------- qbSSP8.05 family I ---------- | | | | |
| 10SH0554 | 3.36 | a | | GG |
| 10SH0553 | 0.68 | b | | AA |

**Table 6.** Heterogeneous inbred families with significant differences for latent period (one out of 22 tested).

| Line | Latent period (days) | | Genotype PZA00910 (qbKSP6.07) |
|---|---|---|---|
| ---------- Parents ---------- | | | |
| B73 | | | CC |
| CML322 | | | TT |
| ---------- qbKSP6.07 family A ---------- | | | |
| 10SH0099 | 5.6 | a | CC |
| 10SH0101 | 2.3 | b | TT |

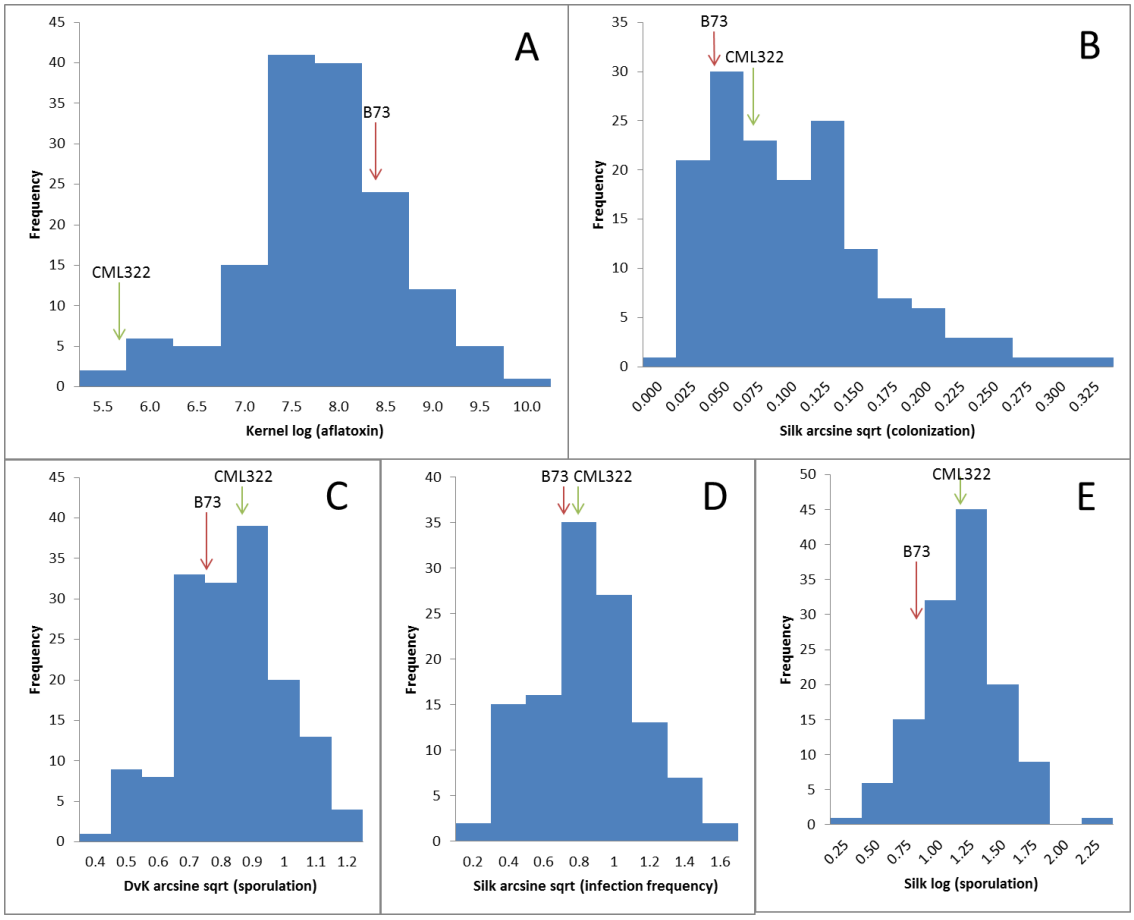


**Figure 1.** Distribution of data for five traits used for QTL mapping. A) Transformed aflatoxin accumulation in field inoculated kernels, CML322 = 5.72, B73 = 8.45. B) Transformed silk colonization in field inoculation experiments, CML322 = 0.07, B73 = 0.03; C) Transformed sporulation on developing kernels for in-vitro inoculation experiments, CML322 = 0.88, B73 = 0.78; D) Transformed silk infection frequency for *in-vitro* inoculation experiments, CML322 = 0.80, B73 =0.77; E) Transformed silk sporulation for *in-vitro* inoculation experiments, CML322 = 1.19, B73 = 0.84.

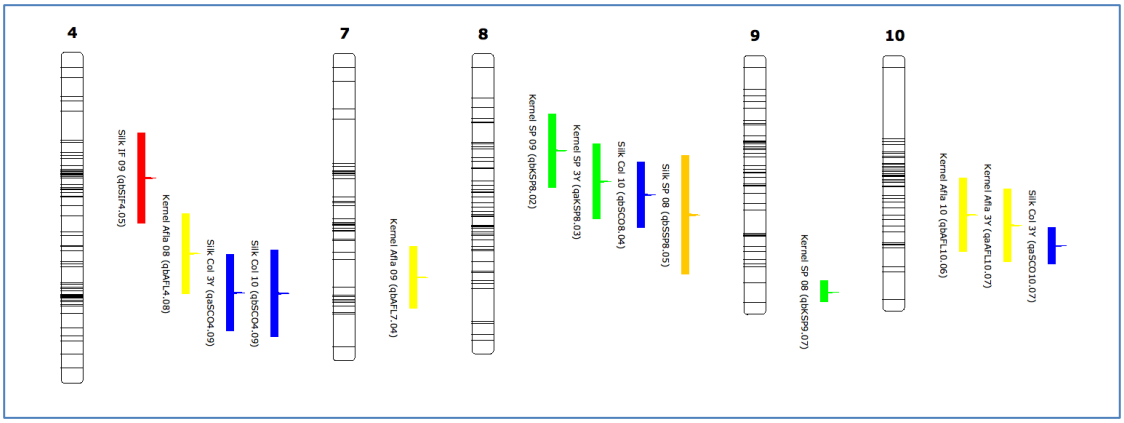


**Figure 2.** QTL locations for five traits analyzed during three years of *in-vitro* and field experiments on the B73 x CML322 population genetic map.

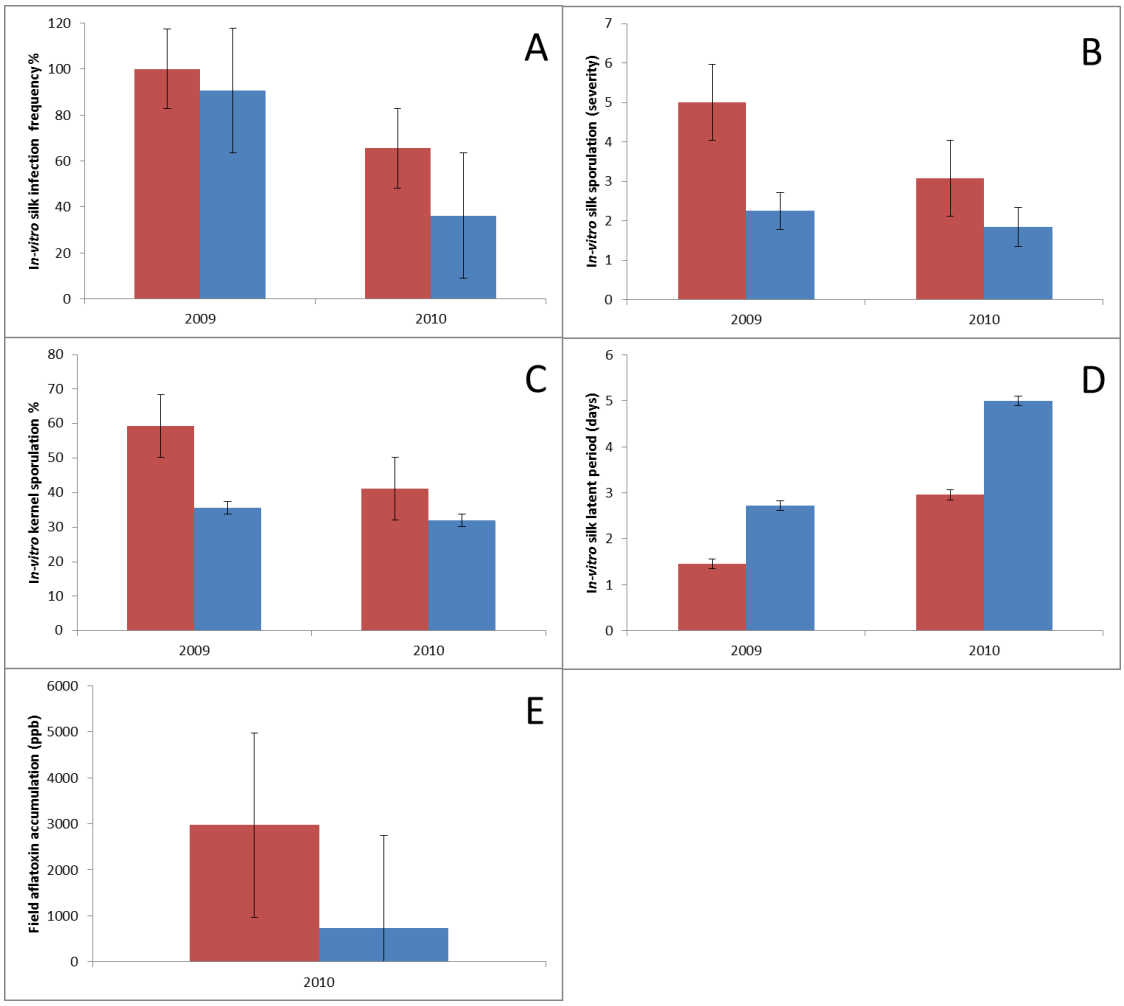


**Figure 3.** Differences between B73 (red bars) and NIL99 [(B73 x TX303)$BC_5S_3$, blue bars] for components of resistance to *Aspergillus flavus*. Error bars represent standard errors. A) *In-vitro* silk infection frequency; ANOVA $p$= 0.85 in 2009 and 0.35 in 2010. B) *In-vitro* silk sporulation; ANOVA $p$= 0.003 in 2009 and 0.304 in 2010. C) *In-vitro* developing kernel sporulation; ANOVA $p$= <0.0001 in 2009 and 0.6459 in 2010. D) *In-vitro* silk latent period; ANOVA $p$ = 0.003 in 2009 and 0.010 in 2010. E) Field aflatoxin accumulation; ANOVA $p$ = 0.041.

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# CHAPTER 5

# META-ANALYSIS OF QTL INVOLVED IN RESISTANCE TO EAR ROT PATHOGENS OF MAIZE

## *Introduction*

Human consumption of food commodities such as maize, wheat and peanut contaminated with mycotoxins results in deleterious effects that include reduced growth and development, impaired immune function, liver failure and death. Aflatoxins are produced by fungi of the genus Aspergillus, especially *Aspergillus flavus*, causal agent of Aspergillus ear rot of maize. Aflatoxin B1 is the most potent naturally-occurring chemical liver carcinogen known. Mutagenesis occurs because a reactive oxygen derivative from the metabolism of aflatoxins in the liver binds to DNA, causing transversions and base substitutions. Aflatoxicosis caused by the ingestion of high doses of aflatoxin causes liver damage. Consumption of contaminated maize has led to periodic outbreaks of fatal aflatoxicosis. Chronic exposure to aflatoxins has been associated with immunosupression and growth impairment in children (33). Other mycotoxins commonly found in maize are fumonisin produced by *Fusarium verticillioides* (teleomorph: *Gibberella fujikuroi* complex), causal agent of Fusarium ear rot of maize, and deoxynivalenol (DON) produced by *Fusarium graminearum* (teleomorph: *Gibberella zeae*), causal agent of

Gibberrella ear rot. There is strong evidence that fumonisins cause esophageal cancer while DON produces nausea and vomiting (24).

Aflatoxin accumulation in maize occurs in the field (pre-harvest) and in storage (post-harvest). Fumonisin and DON accumulation occur mainly before harvest. This study deals only with pre-harvest resistance to mycotoxins, with emphasis on alfatoxins. There are numerous reports of significant variation in the levels of aflatoxin accumulation among distinct maize lines (3, 13, 34, 36). The genetic basis of resistance has been studied using diallel crosses and QTL mapping populations. Resistance to aflatoxin accumulation is quantitative with mainly additive genetic effects (3, 6, 13, 35). Reports of broad sense heritability for aflatoxin are as high as 74% (18). However, preharvest aflatoxin accumulation is highly variable. Temperature and humidity strongly affect final aflatoxin contents in maize (37) and it is thought that drought conditions predispose maize to higher levels of contamination. Rain has been reported to have a strong influence on aflatoxin accumulation in cotton seed (9). In Chapter 3, the role of environmental factors as drivers of aflatoxin accumulation in a set of maize inbreds was described. Also in Chapter 3, a significant correlation of flowering time with resistance to aflatoxin acumulation was reported.

As of early 2011, we are aware of nine published and at least two unpublished studies mapping quantitative trait loci (QTL) for resistance to aflatoxin accumulation and other *A. flavus*-related traits (Table 1). Even though some level of resistance to aflatoxin accumulation have been incorporated into commercial hybrids, it is not

enough to provide adequate control (20). This might be due in part to the imprecision of the mapping studies, the lack of confirmation of these QTL and low levels of heritability. Better levels of resistance are available for Gibberella and Fusarium ear rots (20). One study has reported QTL mapping of resistance to Gibberella ear rot and five studies have analyzed QTL for resistance to Fusarium ear rot (Table 1). In addition, in a subset that included the 24 more resistant and 24 more susceptible lines for fumonisin concentration, Robertson-Hoyt et al. (25) also tested aflatoxin accumulation and found significant genotypic and phenotypic correlations between resistances to the two toxins and ear rot ratings for the two diseases. Considering that the infection and toxin accumulation processes are similar, this fact is not completely unexpected.

All studies for resistance to aflatoxin accumulation highlight the issue of year-to-year variability in their results. QTL mapping studies find that different QTL appear in different years for the same populations. It is unclear whether QTL from the same population but found in different years represent a single QTL. Perhaps because of the variation, QTL are located within large confidence intervals. Finally it is possible that mycotoin–related QTL are the indirect result of loci affecting flowering time (Chapter 3).

QTL meta-analysis uses statistical tools to test for co-localization of QTL from distinct studies (11). QTL meta-analysis integrates multiple QTL studies by creating a consensus genetic map, projecting the QTL derived from multiple studies onto that

consensus map, testing how many QTLs best explain the data of multiple studies, and finally clustering multiple QTL into composite or meta-QTL. Veryrieras et al. (29) expanded on this method by allowing more than four QTL to be tested at the same time and integrating the creation of a consensus genetic map with the QTL meta-analysis *per-se* in a Java computer package (29). Meta-analysis of QTL was first used to identify meta-QTL for flowering time in maize (7), and has since been used in multiple other cases. For disease resistance, the methodology has been used to find meta-QTL for resistance to soybean cyst nematode (14), rice blast (2), Fusarium head blight of wheat (16, 17) and recently maize ear rot (39). The ear rot report included only three Aspergillus ear rot studies and did not involve meta-analysis on mycotoxin accumulation (39).

In this study, we wanted to: i) reduce confidence intervals of mycotoxin-related QTLs so that they can be used in crop improvement, ii) test the hypothesis that QTL for resistance to aflatoxin accumulation and other Aspergillus, Fursarium and Giberella ear rot traits co-localize on the maize genome, and iii) determine whether mycotoxin-related resistance QTL co-localize with flowering time QTL. Finally, to confirm the presence of meta-QTL, we selected introgression lines (ILs) from a public source (27), targeting putative meta-QTL. These ILs were used to test components of silk and kernel resistance in field and *in-vitro* inoculation assays.

## *Materials and Methods*

**Synthesis of QTL Studies.** To our knowledge, there are six published QTL mapping studies for aflatoxin accumulation in maize (4, 6, 21, 30-32). In addition, we have access to the original data sets for two additional, unpublished studies for resistance to Aspergillus ear rot and aflatoxin accumulation (Table 1). The unpublished Wilcox study was conducted at Mississippi State from 1997 to 1998 using the same methods as those reported by Brooks et al. (4). The unpublished Mideros study is presented in Chapter 4; in this analysis, we include aflatoxin accumulation data from 2008 and 2009. Because of the similarity of the interaction, as well as previous reports of linkage of resistance to *Aspergillus* and *Fusarium* (25), QTL maps for resistance to Giberella and Fusarium ear rots were also included in our analysis (1, 10, 22, 26). Several of these publications report QTL maps on more than one population (Table 1).

For meta-analysis, we included only studies that reported a genetic map or for which we had access to full datasets. Presumably because of the variation observed from year to year, most of the authors of the QTL publications presented their results on a per-year basis. Some also included an overall analysis based on data from multiple years. QTL meta-analysis was conducted with three data sets: i) only Aspergillus ear rot (AER) studies, using reported QTLs for multiple years and locations (Meta-analysis A), ii) the AER studies, considering each year and location

for each study as an independent data set (Meta-analysis B) and iii) all ear rot QTL mapping studies considering each year and location as an independent data set (Meta-analysis C). For QTL meta-analysis the software 'MetaQTL' was deployed on a UNIX platform as indicated by Veyrieras et al. (29). As suggested by Truntzler et al. (28), if there were overlapping confidence intervals in studies for the same population in the same year, only the QTL with highest contribution to phenotypic variation was included in the analyses.

**Consensus Genetic Maps.** The software 'MetaQTL' uses a weighted least squares strategy to build a consensus genetic map from multiple genetic maps. Several assumptions were necessary to build this consensus map. First, it is expected that the genetic maps be from independent populations. For Meta-analyses B and C, even though the populations used in multiple years are the same, we assumed independence because QTL results were different, possibly due to environmental variation. Second, it was assumed that there is no recombination interference. Third, it was assumed that the true marker order and recombination rate are the same in the different populations. Maps were inverted, if necessary, to align chromosomes. Finally, it was assumed that all genetic maps share some common markers (29). One exception using a different marker type is explained below.

Using the "InfoMap" command in 'MetaQTL', markers whose order was not consistent between publications where identified (28, 29). Sixteen markers with

inconsistent positions among genetic maps were eliminated for Meta-analysis A and B and 30 markers were deleted for Meta-analysis C. An xml version of the Genetic2008 map downloaded from http://www.maizegdb.org (15), which contained only the markers that were present in any of the five studies included in Meta-analyses A and B, was created using the A2Xml command. Using this auxiliary map, the SNP-based map reported in Chapter 4 was joined with the map used in the other studies, which were based mainly on SSR and RFLP markers. The command "ConsMap" was used to create a consensus map and calculate the goodness-of-fit value of the consensus map for each chromosome (28, 29).

**QTL Meta-analysis.** The "QTLProj" command projects QTL positions from each study onto the consensus genetic map by scaling the original marker interval into the corresponding interval in the consensus map (29). After projection of QTL, we used the "QTLClust" command, which fits a Gaussian mixture model of various numbers of QTL for each chromosome and uses five model selection criteria to return the number of QTL that provided the best results. As suggested by Truntzler et al. (28), QTL confidence intervals used for our analysis were conservative: we used the largest value among those reported or the calculation derived from the $R^2$. For model selection, we also chose the Akaike Information Criterion except in the rare case that most of the other criterion values were different. Finally, the command "QTLModel" creates a file that contains the consensus genetic map, the projected QTL from each study and the meta-QTL on each chromosome.

In order to generalize the results identified by 'MetaQTL', we report the molecular markers closest to the confidence intervals of each consensus map and their position on the maize genome RefGen_V1. Using these anchored coordinates for each meta-QTL from Analysis C, we also determined if they included any of 132 SNP markers recently found to be significantly associated with flowering time in maize (5).

**Chromosome Introgression Lines (ILs).** In order to confirm the presence of QTL identified by preliminary synthesis of QTL studies and by our meta-analysis results, we selected chromosome introgression lines (ILs) from the TBBC3 population that carry segments of the Tx303 genome in the B73 genetic background (27). While Tx303 is not a parent used in any of the QTL mapping studies used in the meta-analyses, previous reports indicate that Tx303 is a possible resistance source for aflatoxin accumulation (19). The original TBBC3 lines were created by Szalma et al. (27) from a cross of B73 and Tx303 and are currently at the $BC_3F_{2:3}$ stage. Two of these lines have been further backcrossed and advanced to $BC_4F_3$ (8).

TBBC3 lines with introgressions in bins 1.01, 4.06, 4.07, 6.05 and 7.03 (5, 6, 10, 3, and 4 ILs, respectively) were selected. In addition, the more advanced $BC_4F_3$ lines developed by Chung et al. (8), targeting bins 1.02 and 1.06, were included in the trials. Because the TBBC3 lines have multiple non-target introgressions, other random non-target loci were also indirectly tested. Selected TBBC3 lines were

planted for two years each in New York (NY) for *in-vitro* and in Mississippi (MS) for field inoculation studies.

***In-vitro* Components of Resistance.** Inoculum was prepared by growing *A. flavus* in 20 g of sterile corn kernels (soaked overnight with 10 ml of $H_2O$ overnight before autoclaving) in 500 ml flasks for 12-18 days, followed by washing, with 20 ml of distilled $H_2O$ with 0.2% Tween 20. The conidial concentration was adjusted with a haemocytometer to $1 \times 10^7$ conidia per milliliter.

Silks and developing kernels were inoculated in the laboratory. Samples were taken from field-grown plants that had been hand pollinated. To produce the test tissues, 12 kernels of each maize line were planted in single rows at Cornell Univerisity's Robert Musgrave Research Farm in Aurora, NY. At anthesis, silks of four plants per row were cut at the tip and the ears were covered with shoot bags. The next day, the newly emerged silk was sib or self-pollinated. One day after pollination, the tips of the ears, including the recently pollinated silks, were cut and transported to the laboratory on ice.

Ten silks from each plant were placed in a 100 mm Petri plate without a lid. The plate was placed in a 24 x 24 mm culture tray lined with chromatography paper. In order to keep a constant humidity, 30 ml of water was added to the chromatography paper. Silks were inoculated by adding 50 µl of a conidial suspension of *A. flavus*

isolate NRRL 3357 with $1x10^7$ conidia per ml. Culture trays were placed in an incubator at 30 ˚C in the dark. All trays were observed daily under a dissecting scope. Latent period (LP) was evaluated as the day when the first sporangium with yellow/green coloration was visible.

For developing kernel (DvK) assays, ears were harvested three weeks after pollination and transported to the laboratory on ice. Five DvK were placed in 60 mm Petri plates that were in culture trays lined with moistened chromatography paper as previously described. Kernels were dip-inoculated in a conidial suspension of *A. flavus* isolate NRRL 3357 at $1x10^7$ conidia per milliliter, prepared as explained above for the silk experiments. Sporulation on each kernel was visually rated on a percentage scale 7 days after inoculation using a dissecting microscope.

**Field Components of Resistance.** A field environment conducive to aflatoxin accumulation was used for the field inoculation experiments at the R. R. Foil Plant Science Research Center at Mississippi State University (MSU). Introgression lines were planted in a randomized complete blocks design with three replicates. Each line was planted in 4 m single-row plots spaced 0.97 m apart. For all inoculations, *A. flavus* isolate NRRL 3357 was seeded onto 50 g of sterile maize cob grits with 100 ml of $H_2O$ and incubated at 28°C for 3 weeks. Before adjusting the concentration of the inoculum to $3x10^8$ conidia per ml, the suspension was filtered through four layers of cheesecloth. Ears were double-inoculated seven days after 50% of the silks had emerged on each row. In order to measure components of resistance in the silk and in

the kernels, both sites were inoculated by injecting 1.7 ml of the conidial suspension in the silk channel and 1.7 ml underneath the husk into the side of the top ear.

For determination of silk infection, two ears of each row where collected seven days after inoculation and transported to the lab on ice. Silk samples from the ear channel were collected in 1.2 ml polypropylene Costar cluster tubes (Corning Inc., Corning, NY) and frozen until processing. Colonization levels were determined using Taqman chemistry qPCR as described previously (19). Briefly, total DNA concentration was determined using Picogreen on all the samples. *A. flavus* DNA concentration was determined by comparing to a set of standards included in each PCR plate. Three replicates of the qPCR procedure were conducted for each sample. The colonization value was calculated by dividing the amount of pathogen DNA by the total DNA for each sample.

For aflatoxin determination at harvest, the top ears of each plant in a row were dried at 38°C for seven days. Kernels from each row were ground with a Romer mill (Union, MO), and a subsample of 50 g was used for aflatoxin measurement using the VICAM AflaTest (Watertown, MA).

**Statistical Analysis and QTL Mapping.** Data analysis was conducted in JMP 8.0 (SAS Institute, Cary, NC). LP and aflatoxin data were log transformed while colonization was arcsine square root transformed prior to ANOVA to standardize

variances. Every component of resistance was analyzed both treating every year as a different location and within each location using a mixed effects model in which replicates and location were considered random effects and line a fixed effect. Because a strong environmental effect was observed, QTL mapping was conducted on each year separately.

Locus effects were determined as indicated by Szalma et al. (27). Briefly, a mixed-effects model was fit for each locus, in which the lines with the Tx303 allele were compared to the recurrent parent B73. To refine QTL locations among the linked significant loci, only the locus with the lowest P value was selected. Correlated loci were then identified using a matrix of all ILs and their introgressions. This was done to identify loci that could not be separated because they were present in the same introgression lines and were found to have a significant effect. Statistical tests were conducted for two correlated markers by selecting fixed lines for the second marker and segregating lines for the first marker. If the lines with the introgressed allele were significantly different from those with the recurrent parent allele, a QTL was reported for the first marker.

## *RESULTS*

**QTL Meta-analysis.** Consensus genetic map A was produced using each of the Aspergillus ear rot (AER) studies separately and using only the QTLs identified with

the combined data for multiple years and locations. The map was 1,773 cM long with 989 markers. The goodness-of-fit statistic produced by 'MetaQTL' for each chromosome was high, ranging from 77 to 103. For all the chromosomes, the $\chi^2$-test rejected the null hypothesis of having the same genetic map among experiments. Twenty-two QTL were projected onto the consensus genetic map (Table 2). Our results indicated that the best model for chromosomes 1, 2, 3, 4 and 5 included clusters of QTLs or meta-QTL (Table 3). Ten meta-QTL were identified on chromosomes 1-5. These meta-QTL were given descriptive designations where 'mq' stands for meta-QTL, 'a' indicates Meta-analysis A, the next three letters refer to the trait (e.g. AFL = aflatoxin accumulation), finally the numbers indicate the bin position in the maize genome. For example, mqaAFL1.01 stands for meta-QTL from Meta-analysis A for aflatoxin accumulation in maize bin 1.01. Based on the anchored markers on the RefGen_V1 maize genome, the meta-QTL from analysis A range from 1.4 to 123.7 Mb.

Consensus genetic map B was formed with the studies for aflatoxin accumulation resistance considering each year independently. The map was 1,791 cM long and included 989 markers. The goodness-of-fit value ranged from 302.9 to 1,376 and the $\chi^2$-test rejected the null hypothesis of similar genetic maps among experiments. Thirty-nine QTL were projected onto the consensus genetic map B (Table 4). Clusters of QTLs or meta-QTL were identified in chromosomes 1, 2, 3, 4, 6 and 7. Fourteen meta-QTL were identified that corresponded to regions from 2.2 to

156.7 Mb on RefGen_V1 (Table 5). Two meta-QTL from meta-analysis B overlapped with meta-QTL from meta-analysis A: mqaAFL4.06 with mqbAFL4.07 and mqaAFL4.09 with mqbAFL4.09.

Consensus genetic map C was created with data from all the ear rot studies considering each year independently. The map was 2,222 cM long with 1,521 molecular markers. The goodness-of-fit was also high for all the chromosomes, ranging from 616 to 1,848. The $\chi^2$-test rejected the null hypothesis of similar genetic maps for all the chromosomes. Eighty-one QTL were projected on consensus genetic map C. Clusters of QTL or meta-QTL were identified on all chromosomes except for chromosome 10 (Table 6, Fig 1). The anchored markers on RefGen_V1 ranged from 1 to 96 Mb for the 36 meta-QTL identified. One meta-QTL (mqcAFL7.02a) could not be anchored to RefGen_V1 because none of the markers located near the confidence interval could be located on the published maize genome. For two meta-QTL (mqcAFL3.06 and mqcAFL3.09), the genetic map positions overlapped but they were recognized as distinct clusters by the program. In addition, three more pairs of meta-QTL had coordinates that overlapped on RefGen_V1 (mqcAFL2.09 with mqcAFL2.10; mqcAFL4.08a with mqcAFL4.08b; and mqcAFL5.06 with mqcAFL5.07). Every meta-QTL from meta-analysis B was contained in one or more meta-QTL from analysis C. In one case, the two analyses produced an identically anchored meta-QTL (mqbAFL6.06 and mqcAFL6.06). In most cases, the meta-QTL from analysis B were represented by more than one meta-QTL of a smaller interval in

analysis C (Table 6). Fifteen out of the 36 (41.6%) meta-QTL found in meta-analysis C contained flowering time QTL (Fig 1).

**Resistance QTL Mapped Using Introgression Lines.** Components of silk and kernel resistance to *A. flavus*, including aflatoxin accumulation in the selected TBBC3 lines were variable between the two years tested. In the silk, B73 was more resistant than Tx303 for *in-vitro* latent period and field colonization in 2009, but differences were not significant in 2008 (Table 7, Fig. 2). For kernel studies, as expected, Tx303 was significantly more resistant than B73 for *in-vitro* sporulation in 2008 and 2009, but significant differences for aflatoxin and colonization were only observed in 2008.

Significant introgression effects were identified for silk and kernel components of resistance (Tables 8 and 9; Fig. 3). Because of the multiple introgressions on each line, some of the significant effects were for groups of correlated markers. For the single independent markers, QTL for silk resistance were identified in maize bins 5.00, 5.04, 7.01, 9.01 and 10.04. For kernel resistance, single markers associated with resistance were identified in maize bins 1.01, 1.03, 4.01, 4.05, 10.03 and 10.04. Among the independent QTL, only the introgression in bin 10.04 was identified for more than one component of resistance (silk latent period and kernel sporulation). No single-introgression QTL were found for kernel resistance in 2008. A large group of correlated markers, covering most of chromosome 2, was associated with resistance to

silk latent period in 2008 and 2009 and for silk colonization in 2009. In addition, a group of correlated markers, including those in maize bins 7.04 and 10.04, was associated with resistance for silk colonization in 2008 and 2009 as well as kernel sporulation in 2008 and aflatoxin accumulation in 2009 (Tables 8 and 9, Fig. 3).

Our QTL analysis using introgression lines was designed to confirm QTLs in five maize bins (1.01, 4.06, 4.07, 6.05 and 7.03), based on the preliminary meta-analysis results. From them, only the introgression region in bin 1.01 was significantly associated with field aflatoxin accumulation, and the resistance was detected only in one year (2009). The marker associated with this introgression line is umc1071. This marker is located between position 7,823,330 and 7,822,522 of chromosome one on RefGen_V1, and is located in our mqaAFL1.01, which does not contain any marker associated with flowering time (Fig. 1).

***Discussion***

Meta-analysis of QTL is based on the assumption that genetic maps obtained from multiple populations are similar. However, it is known that genetic diversity among maize inbred lines is high (12). Therefore it is not surprising that we obtained large goodness-of-fit statistics and that the $\chi^2$-test for every chromosome of the consensus genetic maps created in this study rejected the existence of the same genetic map for all the mapping populations. Similar results have been found in previous QTL meta-analyses that report goodness-of-fit values ranging from 56 to 278 (28).

Another reason for the heterogeneity of the genetic maps is that recombination might vary across different populations. Nonetheless, the consensus genetic maps presented here aligned well to the reference maize genome (RefGen_V1). Another indication that consensus genetic maps are reliable is that only 16 out of 1005 markers had to be removed for meta-analyses A and B and 33 out of 1554 markers for meta-analysis C because of inconsistent positions across studies. In addition, most of these removed markers were in close proximity to each other.

There was little overlap of QTL found for combined multiple-year data and meta-QTL for independent years. Only two pairs of meta-QTL, both on chromosome four, were found in meta-analyses A and B. This result was lower than expected by some authors based on reports of resistance QTL for aflatoxin accumulation, for which there is the sense that the results from independent years are generally in agreement with those of multiple years (31). In this study, we showed that these are mainly exceptions (e.g. those found on chromosome 4) and that the use of multi-year averages for QTL mapping for this highly variable trait can lead to erroneous conclusions on the position of the QTL. It is evident from this analysis that environmental factors have a predominant effect in the expression of QTL for resistance to Aspergillus ear rot.

An interesting feature of meta-analysis is the reduction of confidence intervals on meta-QTL compared to the original QTL. This occurs because in regions where more than one QTL is reported, the meta-QTL is projected only in the area covered by

two or more QTL. Projected QTL covered 28.9, 46.8 and 81.5% of consensus maps A, B and C, respectively, while meta-QTL covered only 16.3, 21.4 and 37.2 %. The meta-QTL are each represented by two or more QTL and reduced average confidence intervals by factors of 1.7, 2.19 and 2.19 for our three analyses. Smaller confidence intervals make the use of this information for breeding purposes more likely.

Meta-QTL are represented by QTL for multiple traits for the same disease and for multiple diseases. For instance mqcAFL1.03 represents a QTL for resistance to Gibberella ear rot disease severity, aflatoxin accumulation and Fusarium ear rot score. At this meta-QTL, we also find co-localization of QTL for toxin accumulation and disease severity symptoms. Because maize inbred lines vary widely with respect to flowering time, and ear rot diseases develop after flowering, this could be a significant source of variation that is difficult to control, especially in field experiments. More than half the mycotoxin meta-QTL contained known QTL markers associated with flowering time. Locus mqcAFL1.03, which consolidated QTL for the three diseases, also contained flowering time QTL and thus it is possible that the effect on multiple ear rots is the indirect result of plant maturity.

Xiang et al. (39) identified meta-QTL for resistance to ear rot in maize but did not analyze mycotoxin traits. Because they did not anchor their meta-QTL to the physical maize genetic map, it is difficult to make direct comparisons with our results. However, they highlighted meta-QTL on chromosomes 3 and 4 because they have smaller confidence intervals and because of they represented a high number of original

QTL. On chromosomes 3 and 4, we also find several meta-QTL with confidence intervals of less than 20 cM on the consensus map and less than 20 Mb on RefGen_V1. In addition, mqcAFL3.06, mqcAFL4.03 and mqcAFL4.09 do not include flowering time QTL.

To confirm the existence of meta-QTL, we selected lines from an unrelated population with introgressions in bins 1.01, 4.06, 4.07, 6.05 and 7.03. These maize bins were selected from preliminary meta-QTL analyses. Although the environment in this study significantly affected these lines, we present clear evidence that Tx303 is more resistant than the recurrent parent B73 for kernel traits. However, the introgression lines produced highly variable results over multiple locations for the two years of study. These results suggest the hypothesis that single QTL are highly susceptible to environmental effects. We find support for this hypothesis in multiple areas of this chapter: first in year-by-year differences in QTL maps for all aflatoxin studies; second, in the variation among years for ILs; and third because lines that contain multiple introgressions seem to be the most stable across years and components of resistance. Its is also possible that the effect of every individual QTL is not large enough to be detected with the power of our experiments and therefore multiple introgressions presumably with QTL that have additive effects are the only ones that we find significant. A recent study found that most QTL for resistance to northern leaf blight, another fungal disease of maize, were too small to be scored on their own (23).

Overall, we have used a statistical analysis to show that certain segments of the mize genome are associated with resistance to all ear rots. We have reduced QTL confidence intervals, which could be useful for breeding purposes specially on marker assisted selection and genomic selection. We attempted to demonstrate the existence of these QTL using a non-related population and only found significant levels of resistance in one set of lines in bin 1.01 during one year for aflatoxin accumulation. The marker that had been introgressed in this set of ILs is located is umc1071 contained in mqcAFL1.01. The meta-QTL does not contain known flowering time QTLin the 2.5 Mb of the maize genome and the marker (umc1071) is a glutathione S-transferase 12 (GST). Interestingly, a recent publication found a different maize GST to be associated with resistance to three foliar pathogens (38).

**Table 1** QTL mapping studies for ear rot resistance in maize used for meta-analysis.

| Plant Disease[a] | Reference | Germplasm | Population type | Population size | Component of resistance[b] | Meta-analysis[c] |
|---|---|---|---|---|---|---|
| AER | Widstrom et al. (2003) | GT-MAS:gk(A1) x GT119 | $F_{2:3}$ | 250 | Afl | |
| AER | Paul et al. (2003) | Tex6 x B73 | $F_{2:3}$ | 176 | Afl, ER | |
| AER | Paul et al. (2003) | Tex6 x B73 | $BC_1S_1$ | 100 | Afl | |
| AER | Busboom and White (2004) | B73 x Oh516 | $BC_1S_1$ | 217 | Afl, BGYF, ER | |
| AER | Busboom and White (2004) | (B73 x Oh516) x LH185 | Test Cross | 217 | Afl, BGYF, ER | |
| AER | Wilcox et al. (unpub.) | Mp313E x Va35 | $F_{2:3}$ | 216 | Afl | A, B, C |
| AER | Brooks et al. (2005) | Mp313E x B73 | $F_{2:3}$ | 210 | Afl | A, B, C |
| AER | Alwala et al. (2008) | Mp313E x Sc212m | $F_{2:3}$ | 142 | PKU, PG | |
| AER | Warburton et al. (2009) | Mp717 x NC300 | $F_{2:3}$ | 270 | Afl | A, B, C |
| AER | Warburton et al. (2011) | Mp715 x T173 | $F_{2:3}$ | 225 | Afl | A, B, C |
| AER | Mideros et al. (unpub.) | B73 x CML322 | $F_2S_5$ | 185 | Afl | A, B, C |
| GER | Ali et al. (2005) | CO387 x CG62 | $F_2S_5$ | 144 | KDS, SDS | C |
| FER | Perez-Brito et al. (2001) | 3 x 18 | $F_{2:3}$ | 238 | ER | C |
| FER | Perez-Brito et al. (2002) | 5 x 18 | $F_{2:3}$ | 206 | ER | C |
| FER | Ding et al. (2008) | 87-1 x Zone3 | $F_{8:9}$ | 185 | ER | C |
| FER | Robertson-Hoyt et al. (2006) | GE440 x FR1064 | $BC_1F_{1:2}$ | 213 | ER, Fum | C |
| FER | Robertson-Hoyt et al. (2006) | NC300 x B104 | $F_2S_6$ | 143 | ER, Fum | C |

[a] The ear rots of maize included in our meta-analysis were: Aspergillus ear rot (AER) caused by *Aspergillus flavus*; Gibberella ear rot (GER) caused by *Fusarium graminearum*; and Fusarium ear rot (FER) caused by *F. verticillioides.*

[b] The various resistance components or traits mapped in each study in each study were aflatoxin accumulation (Afl), percentage kernels uninfected (PKU), pollen germination (PG), severity of ear rot (ER), percent bright greenish yellow florescence (BGYF), kernel disease severity (KDS), silk disease severity (SDS), and fumonisin accumulation (Fum).

[c] We conducted three meta-analyses with different sets of data. Meta-analysis A was done with the overall results for each study for resistance to *A. flavus*. Meta-analysis B was conducted with the reported QTLs of each *A. flavus* study. Finally, meta-analysis C was conducted with studies of B plus the other ear rot studies.

**Table 2** Projected QTL on consensus genetic map A created for the *A. flavus* studies using their overall results only.

| Chromosome | QTL name | Position | CI from | CI to | $R^2$ |
|---|---|---|---|---|---|
| 1 | Warburton_2010_6ALL | 21.0 | 13.9 | 33.0 | 0.02 |
| 1 | Willcox_unpb_1ALL | 32.6 | 17.8 | 46.9 | 0.07 |
| 1 | Mideros_unpb_qaf1.03 | 62.8 | | | 0.33 |
| 2 | Mideros_unpb_qaf2.03 | 23.0 | | | 0.29 |
| 2 | Willcox_unpb_2ALL | 94.1 | 83.0 | 99.8 | 0.04 |
| 2 | Mideros_unpb_qaf2.06 | 103.0 | | | 0.27 |
| 3 | Warburton_2010_9ALL | 27.7 | 16.7 | 41.4 | 0.02 |
| 3 | Brooks_2005_afl4ALL | 103.6 | 92.1 | 118.0 | 0.04 |
| 3 | Warburton_2010_1ALL | 113.5 | 88.9 | 122.5 | 0.04 |
| 3 | Willcox_unpb_3ALL | 136.1 | 113.1 | 142.1 | 0.02 |
| 3 | Willcox_unpb_4ALL | 153.6 | 143.6 | 164.5 | 0.03 |
| 4 | Willcox_unpb_5ALL | 98.8 | 86.6 | 113.0 | 0.05 |
| 4 | Mideros_unpb_qaf4.07 | 107.9 | | | 0.26 |
| 4 | Brooks_2005_afl5-2ALL | 121.2 | 113.7 | 129.9 | 0.13 |
| 4 | Brooks_2005_afl5-1ALL | 166.3 | 156.8 | 177.0 | 0.21 |
| 4 | Willcox_unpb_6ALL | 190.0 | 184.3 | 198.3 | 0.15 |
| 5 | Warburton_2010_12ALL | 107.2 | 77.0 | 122.6 | 0.02 |
| 5 | Warburton_2010_8ALL | 148.6 | 145.4 | 154.6 | 0.12 |
| 7 | Warburton_2009_1ALL | 43.7 | 42.7 | 50.2 | 0.02 |
| 7 | Mideros_unpb_qaf7.04 | 114.0 | | | 0.25 |
| 10 | Warburton_2010_5ALL | 73.9 | 65.6 | 99.8 | 0.06 |
| 10 | Mideros_unpb_qaf10.07 | 106.3 | | | 0.30 |

**Table 3** Meta-QTL analysis A (based on *A. flavus* resistance, using the overall results of the contributing studies). Positions indicated are the closest molecular markers and coordinates of the closest confidence interval marker on consensus genetic map A of the maize genome.

| | | Consensus Genetic Map A | | | | RefGen_V1 for CI markers | | |
|---|---|---|---|---|---|---|---|---|
| Chromosome | QTL name | Position | CI from | CI to | bin | Start | End | Mb |
| 1 | mqaAFL1.01 | 32.0 | 17.7 | 46.4 | | | | |
| | | rab30 | mlo1 | bnlg1953 | 1.01 | 6,221,168 | 12,209,110 | 6 |
| 1 | mqaAFL1.03 | 62.7 | 60.2 | 65.2 | | | | |
| | | AY110052 | pzb01662 | pco063726 | 1.03 | 34,478,875 | 41,390,349 | 6.9 |
| 2 | mqaAFL2.00 | 23.0 | 18.1 | 28.0 | | | | |
| | | cl37982_1 | npi239 | cl4178_1 | 2.00 | 1,423,984 | 2,795,395 | 1.4 |
| 2 | mqaAFL2.06 | 102.7 | 99.6 | 105.8 | | | | |
| | | pco084268 | pza01902 | umc1080 | 2.06 | 89,520,517 | 171,586,692 | 82.1 |
| 3 | mqaAFL3.02 | 27.7 | -17.6 | 73.1 | | | | |
| | | bnlg1144 | umc2105 | 73.63 | 3.02 | 1,460,847 | 125,192,807 | 123.7 |
| 3 | mqaAFL3.06 | 120.9 | 75.8 | 166.0 | | | | |
| | | pzb27 | phm15449 | bnl15.20 | 3.06 | 125,077,410 | 188,817,479 | 63.7 |
| 4 | mqaAFL4.06 | 110.9 | 106.4 | 115.4 | | | | |
| | | bnlg2291 | pza01926 | pza00271 | 4.06 | 158,125,912 | 171,613,479 | 13.5 |
| 4 | mqaAFL4.09 | 166.3 | 156.2 | 176.4 | | | | |
| | | cl14668_1 | gpm553 | hcp101b | 4.09 | 226,125,606 | 243,938,016 | 17.8 |
| 4 | mqaAFL4.11 | 190.0 | 181.3 | 198.7 | | | | |
| | | pza00282 | hcp101b | php20608 | 4.11 | 243,932,999 | 247,095,508 | 3.2 |
| 5 | mqaAFL5.08 | 148.0 | 141.9 | 154.1 | | | | |
| | | pza01140 | phi058 | umc1153 | 5.08 | 207,119,780 | 215,801,019 | 8.7 |

**Table 4** Projected QTL on consensus genetic map B created for the *A. flavus* studies using the results of each year separately.

| Chromosome | QTL name | Position | CI[a] from | CI[a] to | $R^2$ |
|---|---|---|---|---|---|
| 1 | Mideros_unpb08_qaf1.03 | 4.7 | | | 0.33 |
| 1 | Warburton_2010_6 | 23.6 | | | 0.04 |
| 1 | Brooks_2005_afl1M02 | 72.9 | 68.4 | 82.3 | 0.04 |
| 1 | Warburton_2009_4MS05 | 125.2 | 121.5 | 133.9 | 0.01 |
| 1 | Wilcox_unpb_10M98 | 132.1 | 125.8 | 143.6 | 0.22 |
| 1 | Brooks_2005_afl2M01 | 185.0 | 173.5 | 191.4 | 0.07 |
| 1 | Wilcox_unpb_7M97 | 212.5 | 193.1 | 239.1 | 0.07 |
| 2 | Warburton_2009_6Tf05 | 75.3 | 64.9 | 102.7 | 0.11 |
| 2 | Mideros_unpb08_qaf2.03 | 83.9 | | | 0.29 |
| 3 | Warburton_2010_7 | 28.8 | 13.4 | 42.9 | 0.07 |
| 3 | Warburton_2010_9 | 43.4 | | | 0.05 |
| 3 | Warburton_2009_5Tf04 | 60.9 | 46.5 | 61.9 | 0.04 |
| 3 | Brooks_2005_afl4M01 | 88.2 | 67.3 | 100.0 | 0.05 |
| 4 | Brooks_2005_afl5-3M02 | 59.0 | 56.3 | 69.8 | 0.11 |
| 4 | Wilcox_unpb_5M97 | 99.9 | 91.6 | 119.5 | 0.07 |
| 4 | Brooks_2005_afl5-2M01 | 120.3 | 114.7 | 127.9 | 0.10 |
| 4 | Brooks_2005_afl5-2M02 | 122.4 | 114.3 | 133.8 | 0.11 |
| 4 | Brooks_2005_afl5-2M00 | 126.6 | 116.3 | 136.6 | 0.11 |
| 4 | Brooks_2005_afl5-2S00 | 126.6 | 114.5 | 145.9 | 0.06 |
| 4 | Mideros_unpb08_qaf4.08 | 133.6 | | | 0.26 |
| 4 | Wilcox_unpb_9M98 | 166.6 | 152.6 | 179.3 | 0.09 |
| 4 | Brooks_2005_afl5-1M00 | 174.6 | 163.8 | 184.3 | 0.21 |
| 4 | Wilcox_unpb_6M99 | 181.3 | 162.3 | 187.7 | 0.11 |
| 4 | Wilcox_unpb_6M97 | 187.7 | 172.9 | 199.8 | 0.09 |
| 4 | Wilcox_unpb_6M98 | 187.7 | 185.9 | 199.1 | 0.08 |
| 5 | Wilcox_unpb_8M97 | 32.9 | 24.9 | 45.1 | 0.10 |
| 5 | Warburton_2010_10 | 79.7 | 63.7 | 101.7 | 0.16 |
| 5 | Warburton_2010_3 | 151.8 | 145.8 | 157.4 | 0.11 |
| 5 | Warburton_2010_8 | 156.2 | 146.3 | 158.2 | 0.09 |
| 6 | Wilcox_unpb_9M97 | 31.0 | 23.2 | 38.2 | 0.06 |
| 6 | Brooks_2005_afl7M00 | 97.6 | 78.7 | 108.1 | 0.08 |
| 6 | Mideros_unpb08_qaf6.06 | 107.7 | | | 0.34 |
| 7 | Warburton_2009_1MS04 | 44.1 | 43.1 | 51.4 | 0.01 |
| 7 | Warburton_2009_1MS05 | 44.1 | 43.1 | 51.4 | 0.01 |
| 7 | Warburton_2009_3MS05 | 92.5 | | | 0.02 |
| 7 | Mideros_unpb08_qaf7.04 | 114.0 | | | 0.35 |
| 8 | Wilcox_unpb_11M99 | 76.1 | 58.1 | 86.8 | 0.08 |
| 10 | Warburton_2010_11 | 15.9 | 13.9 | 25.8 | 0.05 |
| 10 | Mideros_unpb08_qaf10.07 | 108.5 | | | 0.36 |

[a] CI = confidence interval

**Table 5.** Results from meta-QTL analysis B (only *A. flavus* studies using results by year). Positions indicated are closest molecular markers and coordinates of the closest confidence interval (CI) marker on consensus genetic map B of the maize genome.

| | | Consensus Genetic Map B | | | | RefGen_V1 for CI markers | | |
|---|---|---|---|---|---|---|---|---|
| Chromosome | QTL name | Position | CI from | CI to | Bin | Start | End | Mb |
| 1 | mqbAFL1.01 | 5.2 | 0.1 | 10.3 | | | | |
| | | dmt103b | phi056 | umc1292 | 1.01 | 2,022,607 | 5,384,214 | 3.4 |
| 1 | mqbAFL1.04 | 74.0 | 44.8 | 103.2 | | | | |
| | | asg45(ptk) | vp5 | bnlg1884 | 1.04 | 17,596,049 | 91,728,396 | 74.1 |
| 1 | mqbAFL1.06 | 132.0 | 123.1 | 140.8 | | | | |
| | | umc1035 | pza00068 | bnl7.08a | 1.06 | 183,831,232 | 207,123,567 | 23.3 |
| 1 | mqbAFL1.09 | 196.2 | 167.4 | 225.0 | | | | |
| | | pza00339 | AY110159 | pzb01403 | 1.09 | 227,896,232 | 285,274,085 | 57.4 |
| 2 | mqbAFL2.04 | 83.5 | 81.2 | 85.8 | | | | |
| | | AY104214 | pza03142 | pzb00183 | 2.04 | 22,896,855 | 43,923,497 | 21.0 |
| 3 | mqbAFL3.02 | 32.9 | 20.1 | 45.6 | | | | |
| | | pza03212 | bnlg1325 | phm4204 | 3.02 | 5,415,447 | 17,335,986 | 11.9 |
| 3 | mqbAFL3.04 | 65.0 | 39.6 | 90.3 | | | | |
| | | phm13823 | bnlg1647 | pza01396 | 3.04 | 8,153,417 | 164,833,650 | 156.7 |
| 4 | mqbAFL4.03 | 59.0 | 47.0 | 71.0 | | | | |
| | | pza02138 | gpm480 | pza03048 | 4.03 | 9,733,559 | 26,162,524 | 16.4 |
| 4 | mqbAFL4.07 | 125.6 | 121.0 | 130.3 | | | | |
| | | pza03275 | umc66 | pco123260 | 4.07 | 170,127,442 | 177,666,768 | 7.5 |
| 4 | mqbAFL4.09 | 178.6 | 174.4 | 182.9 | | | | |
| | | pco106324 | PCO088312 | hcp101b | 4.09 | 241,722,897 | 243,938,016 | 2.2 |
| 6 | mqbAFL6.01 | 31.0 | 10.8 | 51.2 | | | | |
| | | bnlg249 | bnlg238 | umc65 | 6.01 | 2,440,673 | 104,604,534 | 102.2 |
| 6 | mqbAFL6.06 | 107.1 | 104.4 | 109.8 | | | | |
| | | AY105728 | umc2389 | umc2170 | 6.06 | 156,739,894 | 159,816,325 | 3.1 |
| 7 | mqbAFL7.03 | 72.2 | 41.0 | 103.4 | | | | |
| | | gst23 | umc1978 | pco136752 | 7.03 | 20,955,574 | 153,023,970 | 132.1 |
| 7 | mqbAFL7.04 | 113.8 | 109.6 | 118.0 | | | | |
| | | AY108844 | pco136752 | cl16175_1 | 7.04 | 153,023,970 | 161,994,205 | 9.0 |

**Table 6.** Results from meta-QTL analysis C (ear rot studies using results by year). Positions indicated are the closest molecular markers and coordinates of the closest confidence interval (CI) marker on consensus genetic map C of the maize genome.

| | | Consensus Genetic Map C | | | | RefGen_V1 for CI markers | | |
|---|---|---|---|---|---|---|---|---|
| Chromosome | QTL name | Position | CI from | CI to | BIN | Start | End | Mb |
| 1 | mqcAFL1.01 | 2.5 | -1.6 | 6.5 | | | | |
| | | dmt103b | phi056(tub1) | cl15090_1 | 1.01 | 2,022,607 | 4,491,045 | 2.5 |
| 1 | mqcAFL1.03 | 77.6 | 73.1 | 82.0 | | | | |
| | | AY106736 | bnlg1484 | AY110393 | 1.03 | 34,967,368 | 51,407,926 | 16.4 |
| 1 | mqcAFL1.05 | 115.1 | 105.5 | 124.6 | | | | |
| | | pza03200 | asg30 | umc167a | 1.05 | 61,103,759 | 157,176,044 | 96.1 |
| 1 | mqcAFL1.06 | 142.8 | 136.1 | 149.5 | | | | |
| | | umc1035 | bnlg1057 | bnlg400 | 1.06 | 189,472,433 | 212,637,488 | 23.2 |
| 1 | mqcAFL1.09 | 197.4 | 187.5 | 207.2 | | | | |
| | | phm16605 | umc1955 | kip1 | 1.09 | 235,256,135 | 255,578,330 | 20.3 |
| 1 | mqcAFL1.11 | 234.0 | 220.1 | 248.0 | | | | |
| | | pza03188 | AY110019 | umc1129 | 1.11 | 270,001,597 | 287,309,081 | 17.3 |
| 2 | mqcAFL2.02 | 7.9 | -17.8 | 33.6 | | | | |
| | | bnlg1017 | phi96100 | pzb01233 | 2.02 | 2,818,792 | 5,044,801 | 2.2 |
| 2 | mqcAFL2.03 | 89.6 | 85.2 | 94.0 | | | | |
| | | pza01755 | pza03142 | bnlg1175 | 2.03 | 22,896,855 | 42,960,640 | 20.1 |
| 2 | mqcAFL2.08 | 162.2 | 157.9 | 166.5 | | | | |
| | | bnlg1662 | pza00804 | cl1288_1a | 2.08 | 212,078,520 | 218,269,636 | 6.2 |
| 2 | mqcAFL2.09 | 208.5 | 198.8 | 218.2 | | | | |
| | | bnlg1520 | AY110389 | bnlg469 | 2.09 | 231,190,201 | 233,060,254 | 1.9 |
| 2 | mqcAFL2.10 | 231.2 | 218.6 | 243.7 | | | | |
| | | umc2214 | bnlg1520 | umc2214 | 2.10 | 220,594,039 | 233,060,494 | 12.5 |
| 3 | mqcAFL3.04 | 57.0 | 51.8 | 62.2 | | | | |
| | | nc030 | bnlg1447 | pco081323 | 3.04 | 10,274,096 | 30,701,731 | 20.4 |
| 3 | mqcAFL3.05 | 92.5 | 85.8 | 99.2 | | | | |
| | | pza00828 | zag2 | pza03073 | 3.05 | 133,480,452 | 168,444,020 | 35.0 |
| 3 | mqcAFL3.06 | 116.5 | 110.4 | 122.5 | | | | |
| | | cl35759_1a | bnlg1063 | csu38a(taf) | 3.06 | 172,927,166 | 178,021,981 | 5.1 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 3 | mqcAFL3.09 | 172.8 | 118.7 | 227.0 | | | | |
| | | umc63a | bnlg1350 | umc1136 | 3.09 | 177,466,400 | 228,963,490 | 51.5 |
| 4 | mqcAFL4.03 | 63.9 | 56.3 | 71.4 | | | | |
| | | pza02138 | umc31a | nc004 | 4.03 | 11,329,035 | 13,359,836 | 2.0 |
| 4 | mqcAFL4.06 | 104.0 | 88.2 | 119.7 | | | | |
| | | gpm458 | hda108 | bnlg2291 | 4.06 | 73,306,996 | 168,691,443 | 95.4 |
| 4 | mqcAFL4.08a | 137.2 | 130.4 | 144.0 | | | | |
| | | umc1667 | pco143166 | bnl7.65 | 4.08 | 172,300,959 | 182,985,362 | 10.7 |
| 4 | mqcAFL4.08b | 161.2 | 150.7 | 171.6 | | | | |
| | | cl42326_1 | umc127 | csu178a | 4.08 | 178,890,260 | 201,487,149 | 22.6 |
| 4 | mqcAFL4.09 | 187.4 | 182.0 | 192.7 | | | | |
| | | pco106324 | cl14668_1 | umc1101 | 4.09 | 240,769,430 | 241,805,659 | 1.0 |
| 5 | mqcAFL5.01 | 85.2 | 74.0 | 96.4 | | | | |
| | | cl35669_1 | gpm707 | bnlg565 | 5.01 | 3,534,270 | 8,606,121 | 5.1 |
| 5 | mqcAFL5.03 | 115.7 | 105.1 | 126.3 | | | | |
| | | pza01523 | bnlg105 | cpn1 | 5.03 | 13,853,155 | 30,239,239 | 16.4 |
| 5 | mqcAFL5.04 | 149.5 | 144.0 | 155.1 | | | | |
| | | AY105205 | bnl7.71 | umc1221 | 5.04 | 141,739,980 | 168,079,328 | 26.3 |
| 5 | mqcAFL5.06 | 167.6 | 160.1 | 175.0 | | | | |
| | | mmc0481 | serk2 | pco143014 | 5.06 | 175,458,849 | 207,274,544 | 31.8 |
| 5 | mqcAFL5.07 | 197.9 | 191.4 | 204.4 | | | | |
| | | phi058 | phi048 | umc1072 | 5.07 | 207,119,780 | 209,947,929 | 2.8 |
| 5 | mqcAFL5.08 | 223.8 | 216.6 | 230.9 | | | | |
| | | umc1225 | pza01140 | bnlg386 | 5.08 | 211,442,506 | 215,800,501 | 4.4 |
| 6 | mqcAFL6.02 | 43.3 | 36.3 | 50.3 | | | | |
| | | csu183 | si1 | AY104775 | 6.02 | 83,628,114 | 102,566,352 | 18.9 |
| 6 | mqcAFL6.06 | 111.0 | 106.5 | 115.4 | | | | |
| | | umc138a | umc2389 | umc2170 | 6.06 | 156,739,894 | 159,816,325 | 3.1 |
| 6 | mqcAFL6.07 | 160.5 | 149.6 | 171.4 | | | | |
| | | umc2059 | umc1653 | umc1127 | 6.07 | 166,227,872 | 168,811,242 | 2.6 |
| 7 | mqcAFL7.02a | 61.5 | 39.4 | 83.7 | | | | |
| | | BC399_1400 | BC618_1000 | BC126_580 | 7.02 | | | |
| 7 | mqcAFL7.02b | 177.2 | 172.6 | 181.8 | | | | |
| | | umc5b | AY109968 | umc116a | 7.02 | 101,321,584 | 121,073,757 | 19.8 |
| 7 | mqcAFL7.03 | 196.8 | 188.5 | 205.0 | | | | |
| | | pza02449 | cl7143_1b | umc1251 | 7.03 | 124,598,825 | 151,621,874 | 27.0 |
| 7 | mqcAFL7.04 | 230.8 | 225.3 | 236.2 | | | | |
| | | AY108844 | umc2332 | pco120172 | 7.04 | 158,028,949 | 162,173,496 | 4.1 |
| 7 | mqcAFL7.05 | 242.1 | 240.0 | 244.2 | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | pza01028 | umc245 | cl48276_1 | 7.05 | 162,579,718 | 165,518,425 | 2.9 |
| 8 | mqcAFL8.05 | 81.4 | 73.0 | 89.8 | | | | |
| | | cyc1 | umc1460 | umc2c | 8.05 | 109,479,980 | 132,155,868 | 22.7 |
| 9 | mqcAFL9.07 | 136.5 | 68.5 | 204.6 | | | | |
| | | phm4303 | umc1688 | umc1982 | 9.07 | 92,800,721 | 150,899,845 | 58.1 |

**Table 7.** Means of the components of silk and kernel resistance to Aspergillus ear rot studied in selected introgression lines (ILs).

| | Silk | | | | Kernel | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *In-vitro* LP[a] | | Field Col[a] | | *In-vitro* DvK SP[a] | | Field aflatoxin | | Field Colonization[a] | |
| | 2008 | 2009 | 2008 | 2009 | 2008 | 2009 | 2008 | 2009 | 2008 | 2009 |
| Parental lines | | | | | | | | | | |
| Tx303 | 2.00 | 2.41 | 0.58 | 0.78 | 26.95 | 12.17 | 1333 | 1161 | 0.04 | 0.11 |
| B73 | 2.22 | 1.45 | 0.31 | 0.18 | 67.21 | 59.32 | 11803 | 2221 | 0.65 | 0.10 |
| Tx303-B73 | 0.22 | -0.96 | -0.27 | -0.60 | 40.25 | 47.15 | 10470 | 1059 | 0.61 | -0.01 |
| *P*-value[b] | 0.583 | 0.042 | 0.684 | 0.012 | <0.001 | <0.001 | 0.002 | 0.368 | <0.001 | 0.745 |
| ILs tested | 9 | 27 | 7 | 23 | 9 | 27 | 7 | 19 | 7 | 12 |

[a] Components of resistance studied were latent period (LP), silk colonization (Col), developing kernel sporulation (DvK SP), aflatoxin accumulation and kernel colonization (IC).
[b] Paired comparison between Tx303 and B73 using student's t-test.

**Table 8.** P-values for molecular markers associated with silk resistance to Aspergillus ear rot in selected TBBC3 introgression lines. *In-vitro* inoculations were conducted in New York and field inoculations in Mississippi.

| | | *In-vitro* Latent Peridod | | Field Colonization | |
|---|---|---|---|---|---|
| Bin | Marker | 2008 | 2009 | 2008 | 2009 |
| QTLs (Independent markers) | | | | | |
| 5.00 | m0151 | | 0.0276 | | |
| 5.04 | b1208 | | | | 2.11E-05 |
| 7.01 | m0171 | | | 0.0034 | |
| 9.01 | b1810 | | 0.0232 | | |
| 10.04 | u1589 | | 0.0002 | | |
| Groups of completely correlated markers | | | | | |
| 1.01 | u1071 | | | 0.0091 | |
| 1.04 | b2295 | | | 0.0091 | |
| 8.04 | u1130 | | | 0.0091 | |
| 1.09 | b1720 | | 0.0234 | | |
| 1.10 | UMC107 | | 0.0234 | | |
| 2.04 | b1175 | 0.0005 | 0.0284 | | 3.04E-05 |
| 2.05 | b1887 | 0.0005 | 0.0284 | | 3.04E-05 |
| 2.06 | u1065 | 0.0005 | 0.0284 | | 3.04E-05 |
| 2.07 | u1637 | 0.0005 | 0.0284 | | 3.04E-05 |
| 2.07 | m0271 | 0.0005 | 0.0284 | | 3.04E-05 |
| 2.07 | b1045 | 0.0005 | 0.0284 | | 3.04E-05 |
| 2.07 | u1560 | 0.0005 | 0.0284 | | 3.04E-05 |
| 2.08 | UMC122 | 0.0005 | 0.0284 | | 3.04E-05 |
| 2.09 | u1551 | 0.0005 | 0.0284 | | 3.04E-05 |
| 2.09 | b1520 | 0.0005 | 0.0284 | | 3.04E-05 |
| 4.03 | u2082 | | 0.0135 | | |
| 4.03 | u2176 | | 0.0135 | | |
| 7.04 | u1412 | | | 0.0174 | 0.0003[a] |
| 7.06 | UMC168 | | | 0.0174 | |
| 7.06 | p116 | | | 0.0174 | |
| 10.04 | u1589 | | | 0.0174 | 0.0003 |
| 4.01 | p072 | | | | 0.000132 |
| 9.01 | b1724 | | | | 0.0001 |
| 10.06 | UMC044A | | | | 0.0001 |
| 10.06 | b2190 | | | | 0.000132 |
| 10.07 | u1084 | | | | 0.000132 |
| 10.07 | b1185 | | | | 0.000132 |

[a] More introgression lines were tested in 2009, therefore two loci from this group were no longer correlated.

**Table 9.** P-values for molecular markers associated with kernel resistance to Aspergillus ear rot in selected TBBC3 introgression lines. *In-vitro* inoculations were conducted in New York and field inoculations in Mississippi.

| | | *In-vitro* DvK Sporulation | | Field aflatoxin | | Field colonization | |
|---|---|---|---|---|---|---|---|
| BIN | marker | 2008 | 2009 | 2008 | 2009 | 2008 | 2009 |
| QTLs (Independent markers) | | | | | | | |
| 1.01 | u1071 | | | | 0.0142 | | |
| 1.03 | UMC076 | | 0.0016 | | | | |
| 4.01 | b1318 | | | | | | 0.000148 |
| 4.05 | b1265 | | | | 0.0448 | | |
| 10.03 | UMC155 | | 2.56E-06 | | | | |
| 10.04 | u1589 | | | | | | 0.0201 |
| Groups of completely correlated markers | | | | | | | |
| 1.04 | b2295 | 0.0032 | | | | | |
| 8.04 | u1130 | 0.0032 | | | | | |
| 1.09 | b1720 | 0.0025 | | | | | |
| 1.10 | UMC107 | 0.0025 | | | | | |
| 1.02 | b1429 | 0.0002 | | | | | |
| 1.02 | b1953 | 0.0002 | | | | | |
| 1.03 | UMC076 | 0.0002 | | | | | |
| 7.04 | u1412 | 0.0172 | | | 0.0124[a] | | |
| 7.06 | UMC168 | 0.0172 | | | | | |
| 7.06 | p116 | 0.0172 | | | | | |
| 10.04 | u1589 | 0.0172 | | | 0.0124 | | |
| 4.01 | p072 | | 0.0066 | | | | |
| 9.01 | b1724 | | 0.0066 | | | | |
| 10.06 | UMC044A | | 0.0066 | | | | |
| 10.06 | b2190 | | 0.0066 | | | | |
| 10.07 | u1084 | | 0.0066 | | | | |

[a] More ILs were tested in 2009, therefore two loci from this group were no longer correlated.

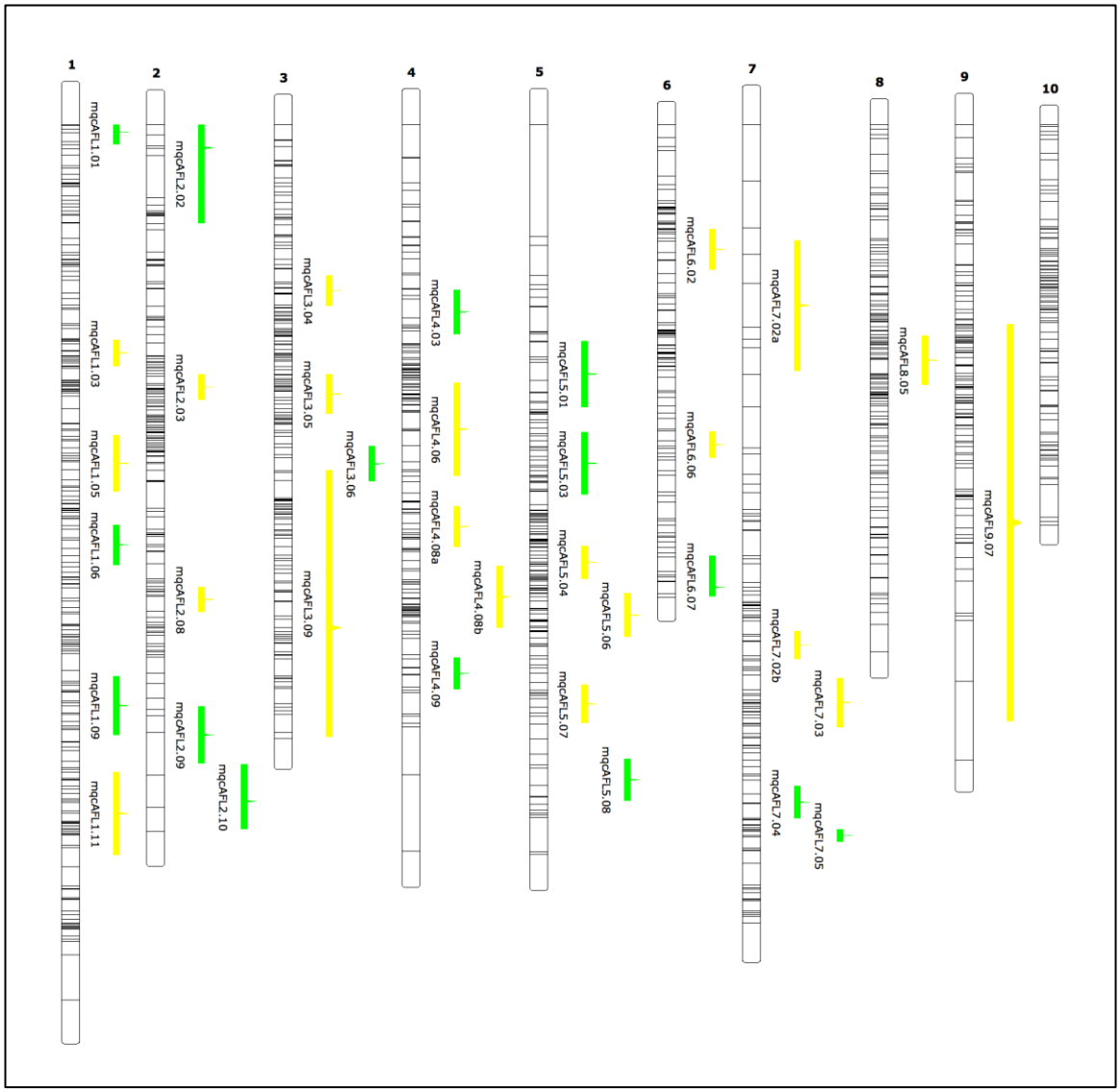


**Figure 1.** Position of meta-QTL on consensus genetic map C. Yellow meta-QTL co-localize with QTL for flowering time as reported by Buckler et al. (5). Green meta-QTL do not contain flowering time QTL based on Buckler et al. (5).

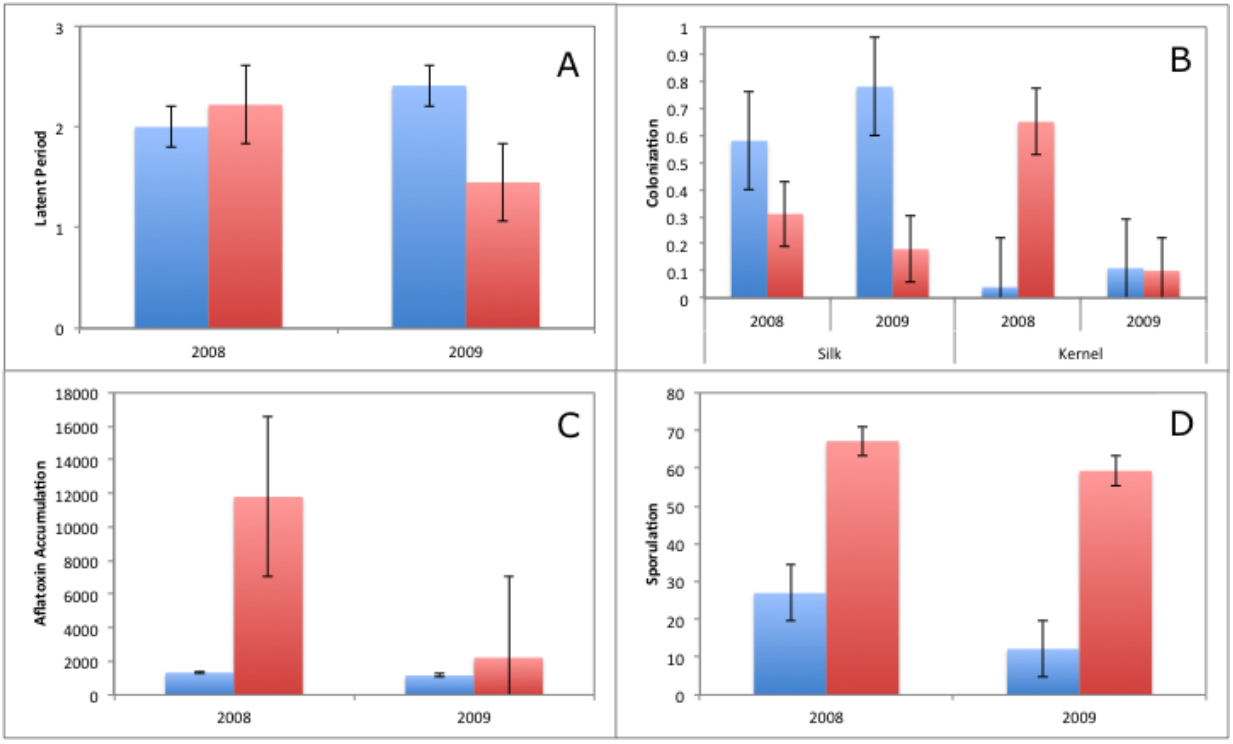


**Figure 2** Comparison of multiple components of resistance to *Aspergillus flavus* between maize inbreds Tx303 (blue bars) and B73 (red). A) Silk latent period after *in-vitro* inoculations. B) Colonization levels by *A. flavus* determined by qPCR after *in-vitro* inoculation of silks and field inoculations of kernels. C) Aflatoxin accumulation levels in ground kernels after field inoculation assays. D) Sporulation on developing kernels after *in-vitro* inoculation assays.

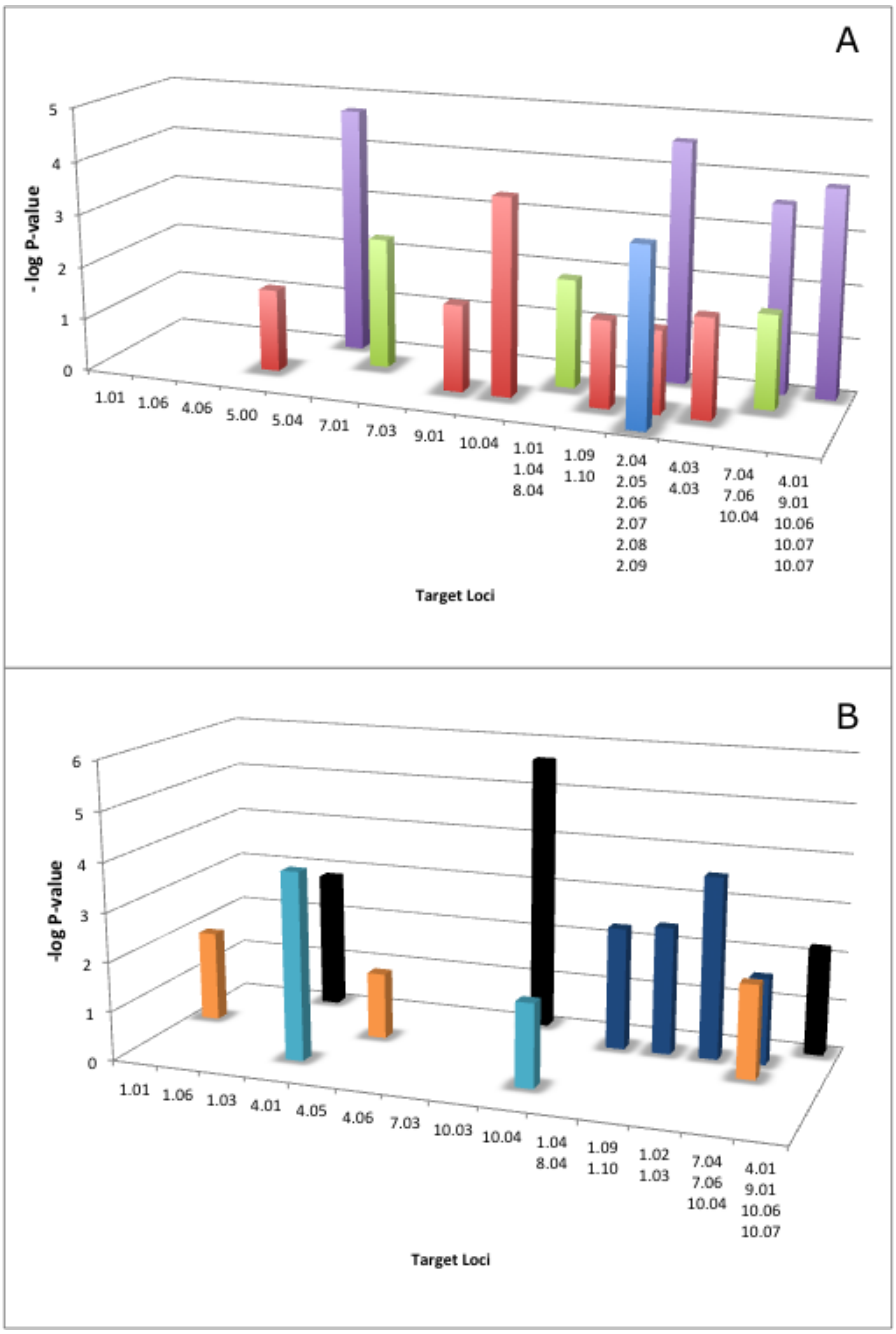


**Figure 3.** Levels of significance (x-axis P-value) for the difference from the opposite allele tested on selected introgression lines for multiple components of resistance. A) Silk components of resistance. Blue bars are for *in-vitro* latent period in 2008 and red bars in 2009. Green bars are for field colonization levels in 2008 and purple bars in 2009. B) Kernel components of resistance. Light blue bars are for colonization in field experiments in 2009; there were no significant differences for colonization in 2008. Orange bars are for aflatoxin accumulation in 2009, there were no significant differences for aflatoxin in 2008. Dark blue bars are for sporulation for *in-vitro* inoculation of developing kernels in 2008 and black bars in 2009.

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